BIOCHEMISTRY OF FRUIT COLOUR IN APPLES

(*Malus pumila* Mill.)

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CONTENTS

ABBREVIATIONS

ANOVA	analysis of variance
BAW	butanol:acetic acid:water
BSA	bovine serum albumin
C_{18}	octadecylsilica
cDNA	complementary DNA
CHI	chalcone isomerase
CHS	chalcone synthase
CIE	Commission Internationale de l'Eclairage
CoA	coenzyme A
Cv(s)	cultivar(s)
DHFR	dihydroflavonol reductase
DHK	dihydrokaempferol
DHQ	dihydroquercetin
DNA	deoxyribonucleic acid
DTE	dithioerythritol
DTT	dithiothreitol
EC	Enzyme Commission
EDTA	ethylene-diaminetetraacetic acid
FW	fresh weight
GT	glycosyltransferase
HPLC	high-performance liquid chromatography
ISCC-NBS	Inter-Society Color Council - National Bureau of Standards
Kat	Katal
K_m	Michaelis constant
LAB	$L^*a^*b^*$ colour space coordinates
L-Phe	L-phenylalanine
LSD	least significant difference
MES	2(*N*-morpholino)ethanesulfonic acid
mRNA	messenger ribonucleic acid
MS	mass spectroscopy
NADPH	nicotinamide adenine dinucleotide phosphate, reduced form
NMR	nuclear magnetic resonance
N.S.	not significant at 5% rejection level
PAL	phenylalanine ammonia-lyase

PC	paper chromatography
PVP	polyvinylpyrrolidone
PVPP	polyvinylpolypyrrolidone (PolyClar AT)
RP	reversed phase
TBA	*t*-butanol:acetic acid:water
TCA	trichloroacetic acid
TFA	trifluoroacetic acid
THF	tetrahydrofuran
TLC	thin-layer chromatography
Tris	tris(hydroxylmethyl)aminomethane
UDP	uridine diphosphate
UV	ultraviolet
λ	wavelength
λ_{max}	wavelength of maximum light absorption

KEY TO TRIVIAL NAMES OF APPLE FLAVONOIDS

Afzelin	kaempferol-3-rhamnoside
Astragalin	kaempferol-3-glucoside
Avicularin	quercetin-3-α-L-arabinofuranoside
Guaijaverin	quercetin-3-α-L-arabinopyranoside
Hyperin	quercetin-3-galactoside
Idaein	cyanidin-3-galactoside
Isoquercitrin	quercetin-3-glucoside
Meratin	quercetin diglucoside
Quercitrin	quercetin-3-rhamnoside
Reynoutrin	quercetin-3-xyloside
Rutin	quercetin-3-rutinoside [L-rhamnose(α1→6)D-glucose]
Taxifolin	dihydroquercetin

LIST OF FIGURES

LIST OF PLATES

LIST OF TABLES

ABSTRACT

Apple fruit display a wide range of colours from green and yellow through to an array of red shades. The biochemical basis of colour differences in apples was investigated with a particular focus on the flavonoids. Three main groups of flavonoids were found: anthocyanins, flavonols and proanthocyanidins. Apples contained predominantly 3′,4′-hydroxylated flavonoids (e.g. cyanidin and quercetin glycosides) with only small amounts of 3′- (e.g. kaempferol glycosides and phloridzin) and 3′,4′,5′-hydroxylated compounds (e.g. (+)-gallocatechin). Galactosides were generally the predominant flavonoid glycosides and the pattern of glycosylation was a result of either the substrate specificity or the levels of specific glycosyltransferases.

Pigment composition was determined for a number of New Zealand-grown apple genotypes and *Malus* species and differences in flavonoids were found to be quantitative and not qualitative. In addition, there were large differences in the concentrations of chlorophyll and carotenoids. Changes in pigment composition, and the activities of three enzymes of the flavonoid biosynthetic pathway (PAL, CHI and GT) were measured during the development of red and non-red apple fruit. The composition of the flavonoids in red and non-red cultivars was similar except that the red cultivar synthesized cyanidin glycosides during ripening. There were significant quantitative changes in all pigment groups during ripening but there were no significant qualitative changes in flavonoids. There was coordinate regulation of the flavonoid enzymes, including PAL, during both developmental regulation and UV induction of flavonoid biosynthesis. Changes in enzyme activities generally correlated with changes in flavonoid concentration, particularly during ripening, although none of these enzymes appeared to be the rate-limiting step(s) in flavonoid biosynthesis. PAL-IS did not have a significant effect in controlling the changes occurring in flavonoid biosynthesis. Differences in accumulation rates of flavonoids in the two cultivars investigated in detail were a function of the levels of enzyme activity.

The colour of an apple fruit was determined by a number of factors. A major control point was one of the final steps in the flavonoid pathway (conversion of leucocyanidin to cyanidin) resulting in the synthesis, or lack of synthesis, of the anthocyanins. In all apple genotypes and cultivars examined the genes encoding for the enzymes responsible for catalysing this step must have been present, but the control was at the level of expression. Copigmentation is the bonding between anthocyanins and other phenolic molecules resulting in stabilization of the anthocyanin in its coloured form, a bathochromic shift and an increase in the absorbance of the visible band. In apples copigmentation probably had a role in stabilising anthocyanin colouration but did not influence colour variation because it was a constant factor. Self-copigmentation may be occurring in some cultivars with high anthocyanin levels, resulting in blueing of the red colour. The intensity of the red colour was a function of the energy requirement for pigment synthesis, vacuolar size, pigment distribution and the influence of environmental factors. The major differences in hue were more likely to be due to the visual blending of chlorophyll, carotenoids and

anthocyanins. Thus, the final appearance of the fruit was the result of the initial pigment concentrations plus the combined changes and hence the resultant final concentration of all three pigment groups (flavonoids, carotenoids and chlorophyll).

The biochemical basis for fruit colour variation in apples has been determined but there are still some gaps in our knowledge particularly the control of flavonoid biosynthesis. The prospects for future genetic manipulation of apple fruit colour are discussed.

Section 1 - INTRODUCTION

1.1 Fruit colour

When fruits ripen they display their colour in a variety of shades that stand out against the contrasting green of the surrounding leaves. The role of these colours is to attract insects and other animals thereby ensuring seed dispersion. Thus, attractive coloration is proposed as a mechanism for propagation of the plant species (Gross, 1987). Gradually, humans discovered that these natural colours were due to plant pigments and today fruit colour is one of the major criteria by which people select their fruit. The appearance, and thus the appeal, of many fruits depends on the type and amounts of various pigments present in the peel tissue and the underlying flesh of the fruit.

A pigment is a chemical substance capable of absorbing visible light due to the action of a chromophore, its colour being complementary to that of the absorbed light (Gross, 1987). There are three groups of pigments responsible for fruit appearance each with a different colour range. The background green and yellow colours are due to the levels of plastid pigments, chlorophyll and carotenoids. In red cultivars these are often masked by the presence of a group of flavonoid pigments, the anthocyanins, that are located in the vacuole (Knee, 1972). When carotenoids and chlorophyll co-occur with the anthocyanins they lead not only to orange-red colours but also bronze colours, which cannot be formed by any of the pigment types alone (Forkmann, 1991). Other types of flavonoids, besides the anthocyanins, are often also present in large amounts in fruit. Together with the carotenoids they may make some contribution to yellow colour although they are thought to be less important (Workman, 1963). Changes in the quantity and kind of flavonoids and anthocyanins will lead to subtle changes in colour hues that add to the appeal of fruit colour. In addition the flavonoids, including the anthocyanins, are developmentally regulated and are also formed in response to stress factors such as high light and temperature (Macheix *et al.*, 1990). Changes in these factors will alter the final appearance of the fruit.

The commercially grown apple - *Malus pumila* Mill. (syn *Malus domestica* Borkh.) - belongs to the family Rosaceae, which is a very large family comprising more than 100 genera and more than 2000 species. Together with the pear (*Pyrus*), apples belong to the subfamily Pomoideae, which contains 18 genera (Rehder, 1940). The family includes many other fruit-producing plants including the apricot, plum, cherry and peach (*Prunus*), strawberry (*Fragaria*), blackberry and raspberry (*Rubus*) and quince (*Cydonia*), as well as the popular ornamental plant, the rose (Harborne, 1967). A wide range of fruit colours are seen within the family including many different shades of red from bright red (cherry), to orange-red (peach) through to dark red-black (blackberry). Even within a species, such as apple, cultivars display many colours from greens and yellows through to a range of red shades, from pink-red or orange red to dark red-purple (Plate 1.1).

Although there has been some work on the identification and quantification of the various pigments

Plate 1.1: Range of colours displayed by apple fruit

that are present in apple skins (Walker, 1964; Burda *et al.*, 1990; McRae *et al.*, 1990; Mazza & Velioglu, 1992), the contribution they make to the final appearance of the fruit is not fully understood. Details of the particular compounds present and quantification of these, particularly in New Zealand-grown apple cultivars, is required to complement existing knowledge. It is also unclear whether other factors, such as copigmentation, play a role in apple fruit colour and some of these require investigation. Study of the developmental changes and the biosynthesis of these pigments also provides useful information on how the final fruit colour is achieved. Thus, this thesis will determine the biochemical basis of apple fruit colour with a particular focus on one group of pigments - the flavonoids.

1.2 The flavonoids

It is estimated that about 2% of all carbon photosynthesized by plants is converted into the various flavonoid groups such as the anthocyanins (Smith, 1972). Thus, flavonoids constitute one of the largest groups of naturally occurring phenols and are virtually ubiquitous in green plants (Markham, 1982). In plants, flavonoids occur in a variety of structural forms; all contain 15 carbon atoms in their basic nucleus and these are arranged in a C_6-C_3-C_6 configuration, that is two aromatic rings (labelled A and B) linked by a three carbon chain (Markham, 1982). This chain is closed in most flavonoids, forming the heterocyclic C ring, but remains open in the chalcones and dihydrochalcones (Stafford, 1990). Natural flavonoids are divided into subclasses based generally on the oxidation state of the C-ring (Table 1.1). For convenience, the individual carbon atoms on each of the rings are referred to by a numbering system that uses ordinary numerals for the A- and C-rings and "primed" numerals for the B-ring (Figure 1.1). However, a modified numbering system is used for chalcones with primed numerals for the A-ring and ordinary numerals for the B-ring. Aurones are also numbered differently due to having one less carbon atom on the C-ring.

The flavonoid variants are related by a common biosynthetic pathway that incorporates precursors from both the "shikimate" and "acetate-malonate" pathways (Heller & Forkmann, 1988), the first flavonoid being produced immediately following confluence of the two pathways (Markham, 1982). The flavonoid formed initially in the biosynthesis is the chalcone and all other forms are derived from this by a variety of routes (Figure 1.2). Details of the biosynthesis of the groups important in apples are discussed in Section Five. Further modification of the flavonoid may occur at various stages resulting in additional (or reduced) hydroxylation; methylation of hydroxyl groups, or of the flavonoid nucleus; isoprenylation of hydroxyl groups, or of the flavonoid nucleus; methylenation of *ortho*-dihydroxyl groups; dimerization; bisulphate formation; and, most importantly, glycosylation of hydroxyl groups to produce flavonoid *O*-glycosides, or of the flavonoid nucleus to produce flavonoid *C*-glycosides (Markham, 1982). There are thus a great number of flavonoids known with varying distribution amongst species and even amongst

Table 1.1: The main classes of flavonoid aglycones and their structures (adapted from Swain, 1965)

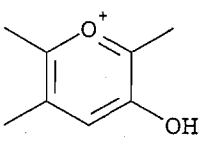
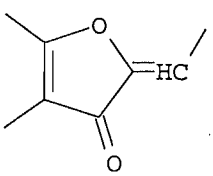
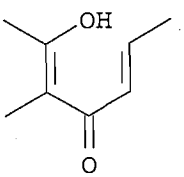
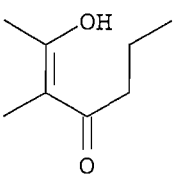
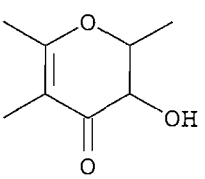
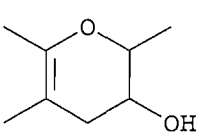
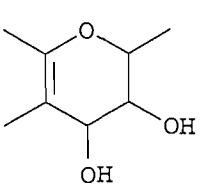
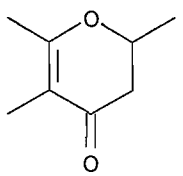
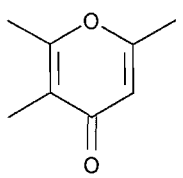
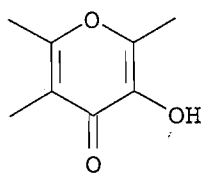
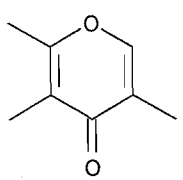
Name	Structure of C-ring	Typical members and position of hydroxyl groups
Anthocyanidins		Pelargonidin; 3,4,7,4′ Cyanidin; 5,7,3′,4′ Delphinidin; 3,5,7,3′,4′,5′
Aurones		Sulphuretin; 6,3′,4′ Aureusidin; 4,6,3′,4′
Chalcones		Chalconaringenin; 2′,4′,6′,4 Butein; 3,4,2′,4′
Dihydrochalcones		Phloretin; 4,2′,4′,6′ Hydroxyphloretin; 3,4,2′,4′,6′
Dihydroflavonols		Fustin; 7,3′,4′ Taxifolin; 5,7,3′,4′
Flavan-3-ols		(+)-Afzelechin; 5,7,4′ (+)-Catechin; 5,7,3′,4′ (+)-Gallocatechin; 5,7,3′,4′,5′
Flavan-3,4-diols (Leucoanthocyanidins)		Leucocyanidin; 5,7,3′,4′ Leucodelphinidin; 5,7,3′,4′,5′

Table 1.1: *continued*

Name	Structure of C-ring	Typical members and position of hydroxyl groups
Flavanones		Pinocembrin; 5,7 Naringenin; 5,7,4′ Eriodictyol; 5,7,3′,4′
Flavone		Chrysin; 5,7 Apigenin; 5,7,4′ Luteolin; 5,7,3′,4′
Flavonols		Galangin; 5,7 Kaempferol; 5,7,4′ Quercetin; 5,7,3′,4′ Myricetin; 5,7,3′,4′,5′
Isoflavones		Genistein; 5,7,4′

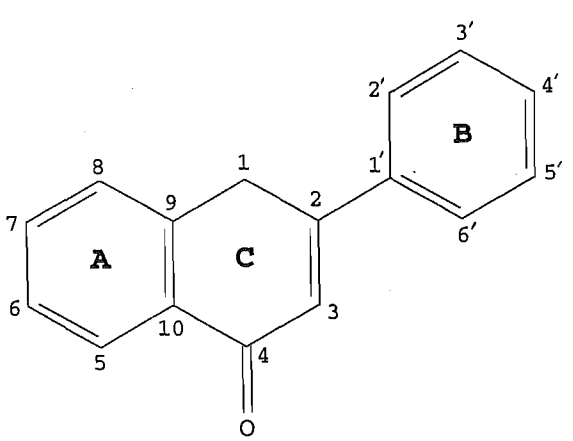


Figure 1.1: Structure and numbering of the flavonoid nucleus

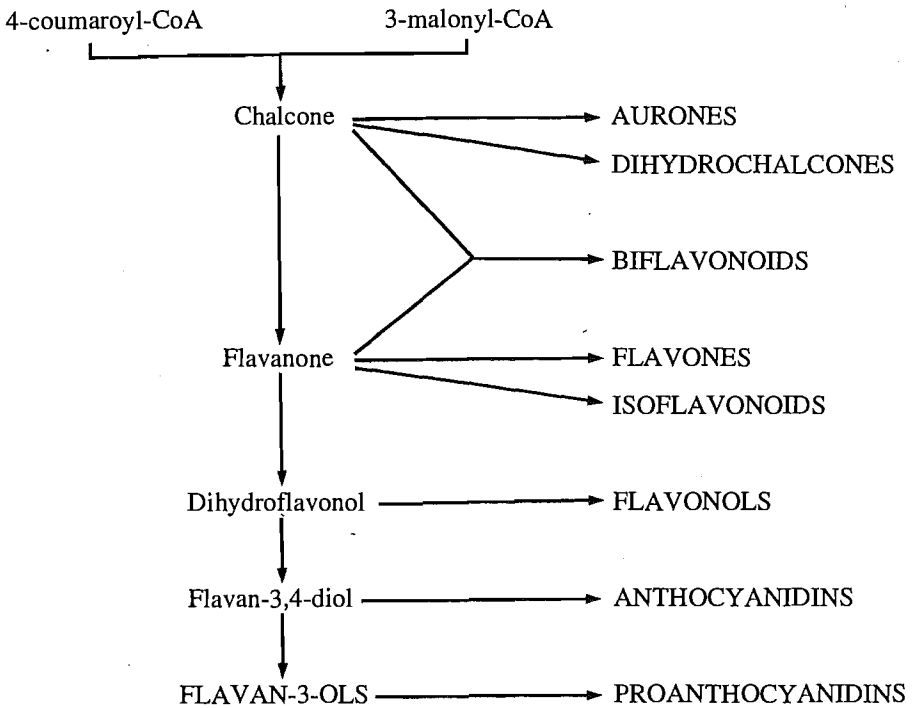


Figure 1.2: Overall pathway to the main flavonoid groups

different tissues types of the same plant.

There is a range of hydroxylation patterns seen among the flavonoids but some patterns are observed more frequently. The 5,7-hydroxylation pattern of the A-ring is the most common one although a 7-hydroxyl ring (generally called a 5-deoxy ring) is common in the isoflavonoid subgroups and in some proanthocyanidins. Occasionally a 5,7,8- or 5,6,7-hydroxylation pattern is found. The B-ring generally has a 4′-, a 3′,4′ or a 3′,4′,5′-hydroxylation pattern. Some rare flavonoids lack B-ring oxidation and in the isoflavonoids a 2′-hydroxylation pattern is found. For the C-ring the most frequent hydroxylation pattern is at the carbon '3' position (Stafford, 1990).

In the plant flavonoids commonly occur as flavonoid *O*-glycosides in which one or more of the flavonoid hydroxyl groups is bound to a sugar by an acid-labile acetal bond (Markham, 1982). The effect of glycosylation is to make the flavonoid less reactive and more water (sap) soluble, which allows the storage of the flavonoids in the cell vacuole where they are commonly found (Harborne, 1967). Although any of the hydroxyl groups on the flavonoid nucleus may be glycosylated, some have a higher probability of being so than others. Flavones, isoflavones and dihydroflavones are usually glycosylated in the 7-hydroxyl position, for the flavonols and dihydroflavonols the 3- (and 7-) hydroxyl is glycosylated, and for the anthocyanins it is the 3- (and 5-) hydroxyl. Glucose is the most common sugar to be attached with galactose, rhamnose, xylose and arabinose also relatively common. A number of other sugars are

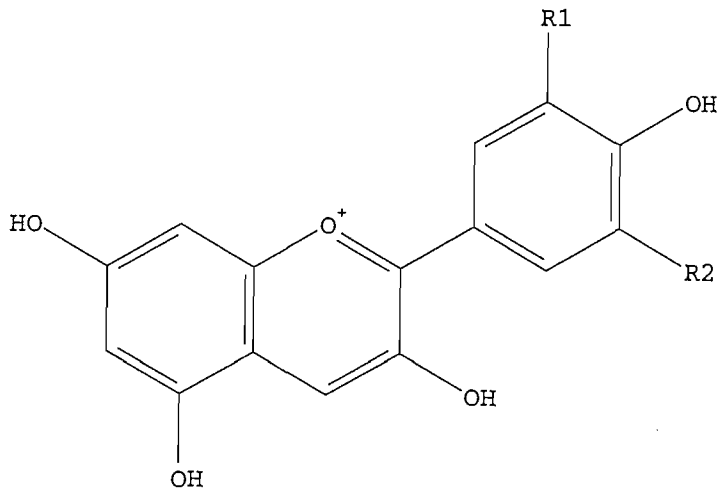
encountered occasionally and disaccharides, such as rutinose (6-*O*-α-L-rhamnosyl-D-glucose), are also frequently found in association with the flavonoids. Glycosides may exhibit a further modification, which is acylation of one (or more) of the sugar hydroxyls with an acid such as acetic or ferulic (Markham, 1982). The acid effectively esterifies the sugar due to the formation of an ester bond. The distribution of these compounds is more restricted than the standard glycosides (Harborne, 1986).

Flavonoids are characteristic constituents of green plants with the exception of algae and hornworts. They occur in virtually all plant parts including leaves, roots, wood, bark, pollen, nectar, flowers, berries and seeds (Markham, 1982). There is a general trend towards an increased range of flavonoids being present in the more highly evolved plant groups and taxonomically related plants generally also produce similar types of flavonoids (Harborne, 1972). Thus, useful information about flavonoid types likely to be present in a particular plant can be obtained by reference to literature on studies of related plants such as those from the same genus or family.

Three classes of flavonoids are very widespread in fruits and are quantitatively dominant: anthocyanins, flavonols and proanthocyanidins. The latter class is present as monomers and as condensed forms (tannins). Other classes (in particular flavones, flavanones, flavanonols or dihydroflavonols, chalcones and dihydrochalcones) are quantitatively less important except in certain special cases such as *Citrus* (Macheix *et al.*, 1990). Diversity and complexity of the flavonoids of fruits depends on at least two factors: firstly the variety of aglycones and the high number of glycosides possible, sometimes in acylated form, and secondly their association into complex molecules (Gross, 1987).

1.2.1 Anthocyanins

The term anthocyanin (from the Greek *anthos*, flower and *kyanos*, blue) was first used by Marquart in 1835 to denote the blue pigment of cornflower and later was used to define the whole group of related pigments (Hayashi, 1962). The anthocyanins (glycosylated anthocyanidins) are particularly characteristic of the angiosperms and, apart from a few reports in ferns and mosses, are not found elsewhere in nature (Harborne, 1967). Anthocyanins are the pigments that give most flowers and fruits their pink, red, violet or blue colours although in some cases, such as the tomato, anthocyanins are absent and the red colour is due to the presence of certain types of carotenoids (Macheix *et al.*, 1990). The anthocyanidins are all based chemically on a single aromatic structure - that of the 3,5,7,3′,4′ pentahydroxyflavylium cation, cyanidin (Harborne, 1967). There are six anthocyanidins that are widely distributed in plants: pelargonidin, cyanidin, peonidin, delphinidin, petunidin and malvidin (Figure 1.3). Anthocyanidins other than these six common ones are few in number and very rare in occurrence (Harborne, 1967). These six anthocyanins differ in the number of hydroxyl groups on the B-ring and the presence or absence of methyl groups that affect the colour (Gross, 1987). The colour of anthocyanins is also affected by the nature and number of sugars attached to the molecule, the position of the attachment, the nature and number of the aliphatic or aromatic acids attached to the sugar and the physiochemical medium in which they occur



Pelargonidin	R_1=R_2=H
Cyanidin	R_1=OH, R_2=H
Peonidin	R_1=OMe, R_2=H
Delphinidin	R_1=OH, R_2=OH
Petunidin	R_1=OMe, R_2=OH
Malvidin	R_1=OMe, R_2=OMe

Figure 1.3: Structures of the common anthocyanidins

(Mazza & Brouillard, 1990). The colours of the main anthocyanins under standard conditions (0.01% HCl in methanol) vary from the scarlet-red of pelargonidin through to the crimson of cyanidin and the blue-mauve of delphinidin, with absorption maxima of 520, 535 and 546 nm respectively (Gross, 1987). However, in the intact plant cell these colours vary depending on a number of factors such as pH, copigmentation and the presence of other factors. These are discussed in more detail in Section Three.

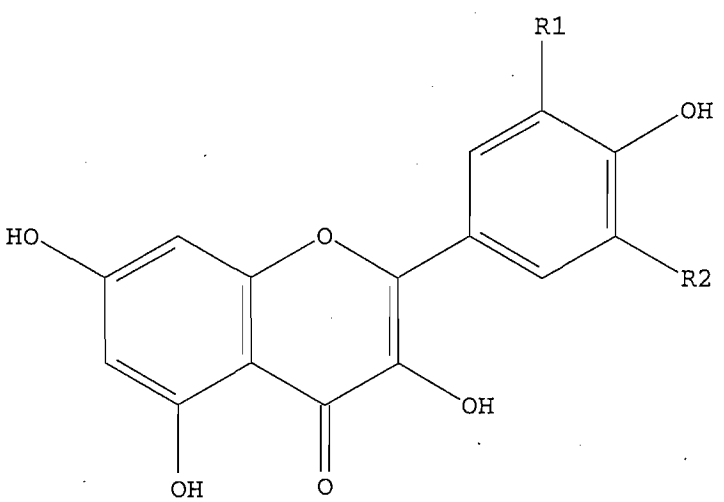
Anthocyanin chemistry has provided a considerable challenge because of difficulties in isolating them and also because of their relative lability. Although the anthocyanins, which have sugars attached to them, are sap-soluble and reasonably stable, the aglycones are insoluble in water, unstable to light and rapidly destroyed by alkali (Harborne, 1967). Despite this, much work has been carried out on anthocyanins, particularly in flowers, and a large range of different structures have been reported in many reviews (Hayashi, 1962; Harborne, 1967; Timberlake & Bridle, 1975; Timberlake, 1981; Hrazdina, 1982). In fruits, the variation in structure is more limited than in flowers and a study of 40 fleshy fruits showed that cyanidin was the most common anthocyanidin - it was present in 90% of fruits examined (Macheix *et al.*, 1990). Very few red fruits do not contain cyanidin (Gross, 1987).

The anthocyanin pattern of the Rosaceae is distinct in many respects, the most striking being the complete absence of delphinidin or its derivatives (Harborne, 1967). Cyanidin is a very common pigment whilst pelargonidin is relatively rare, however the glycosidic patterns vary considerably within the genus.

Two of the Pomoideae, *Malus* (apple) and *Pyrus* (pear), both contain cyanidin 3-galactoside, which is generally absent from other subfamilies (Harborne, 1967). Details of the specific anthocyanins found in apples are discussed in Section Two.

1.2.2 Flavonols

Flavonols differ from the anthocyanins in being more highly oxidized. From the point of view of colour, anthocyanins are, in general, much more important. However, in some plants, flavonols do make a significant contribution, either as yellow pigments in their own right, or as copigments to anthocyanins, stabilising them and having a blueing effect (Harborne, 1967). Flavonol glycosides often correspond closely in structure with anthocyanins. The two groups are frequently found together and are intimately related biosynthetically (Stafford, 1990). Three flavonols are common in plants: kaempferol, quercetin and myricetin (Figure 1.4) (Harborne, 1967). In fruits, a fourth aglycone isorhamnetin (3′,4′-Me quercetin) is also often present (van Buren, 1970). Flavonols, like anthocyanins, occur in living tissue bonded to a sugar(s) and a large number of different flavonol glycosides have been described (Wollenweber & Jay, 1988). Most common flavonol glycosides are similar in structure to the anthocyanins and include 3-glucosides, 3-galactosides, 3-rutinosides and 3-sophorosides (Harborne, 1967). Flavonols differ from anthocyanins in being structurally more variable and many more flavonols are known, both aglycones and glycosides (Harborne & Williams, 1975).



Kaempferol	R_1=R_2=H
Quercetin	R_1=OH, R_2=H
Myricetin	R_1=R_2=OH
Isorhamnetin	R_1=OCH_3, R_2=H

Figure 1.4: Structures of the common flavonols

There is considerable structural variation in the nature of the anthocyanins and flavonols present in the plant particularly in the patterns of hydroxylation and glycosylation. For example in the strawberry (*Fragaria* sp.) pelargonidin and kaempferol-3-glucosides are predominant. These compounds both have the same hydroxylation pattern with only the 4′-hydroxyl on the B-ring (Macheix *et al.*, 1990). For some species, the hydroxylation of the anthocyanins and flavonols differ, such as in sweet cherry (*Prunus avium*) where cyanidin (3′,4′-OH) and peonidin (3′-OMe, 4′-OH) glycosides are dominant yet kaempferol glycosides (4′-OH) make up a large percentage of the flavonols (Macheix *et al.*, 1990). The hydroxylation patterns of the anthocyanins and flavonols are the same in some species but the composition of the glycosides differs. In the raspberry (*Rubus idaeus*) cyanidin-3-sophoroside is the major anthocyanin in most cultivars yet the sophoroside is not present in the flavonols (Macheix *et al.*, 1990).

The most striking fact about the flavonoid pattern of the Rosaceae is the almost complete absence of substances with 3′,4′,5′-trihydroxylic substitution. Kaempferol and quercetin are ubiquitous in the family with myricetin being present in only a few species (Harborne, 1967). A number of other flavonol glycosides have been identified in some genera and the glycosidic pattern is very variable. A survey of a number of *Malus* species showed both quercetin and kaempferol glycosides present in the leaf and bark (Williams, 1982). In addition, glucosides of isorhamnetin and azaleatin (5-Me quercetin) are common in some species (Williams, 1982) and galangin has also been detected (Giannasi, 1988). However, in the domestic apple quercetin is the predominant flavonol in all plant parts, including the skin, with kaempferol being present in smaller amounts (Williams, 1960) and with some variation in the range of glycosides found. Other compounds present in some *Malus* species were not detected in either the leaf or the bark of *Malus pumila* (Williams, 1982). The details of the particular flavonol glycosides present in apple skin, and the distribution in other plant parts are discussed in Section Two.

1.2.3 Proanthocyanidins and related compounds

A third group of flavonoids are present in fruits and these are a diverse group often collectively named the proanthocyanidins. There has been a great deal of confusion over the names of the various compounds included in this group, and care has to be taken, when reviewing the literature, about which compounds are being referred to. Proanthocyanidins were designated by Fredenberg and Weinges (1960) as all the colourless substances isolated from plants that form anthocyanidins when heated with acid. The name proanthocyanidin is a chemical and not a biochemical term and it does not imply any biogenetic relationship (Haslam, 1975). Weinges *et al.* (1969) reserved the term leucoanthocyanidin for the monomeric proanthocyanidins such as the flavan-3,4-diols. The flavan-4-ols are also now classed in this group (Porter, 1988). Leucoanthocyanidins now include all monomeric flavonoids that produce anthocyanidins by cleavage of a C-O bond on heating with mineral acid (Porter, 1988). They are frequently found in the wood and bark of trees but are not major compounds in fruits (Macheix *et al.*, 1990). Condensed proanthocyanidins was the name used for the various flavan-3-ol dimers and higher

oligomers (Weinges *et al.*, 1969). The term 'condensed' has now been dispensed with and the group has been redefined to include all compounds that produce anthocyanidins by cleavage of a C-C bond (Porter, 1988). Included in the proanthocyanidins is a further group, the natural flavan-3-ols, which are the most common (Porter, 1988). In nature, the most widely distributed members of this class of phenolic flavan-3-ols are the diastereoisomeric pair (+)-catechin and (-)-epicatechin (Figure 1.5). These share a distribution almost as widespread as quercetin, the flavonol relation with the equivalent hydroxylation pattern (Porter, 1988). A frequently recognized pattern of proanthocyanidins is the co-occurrence of (-)-epicatechin with procyanidin B-1, B-2 and C-1 (Haslam, 1982). These procyanidins are dimers, and a trimer, composed of (+)-catechin and (-)-epicatechin units (Figure 1.6).

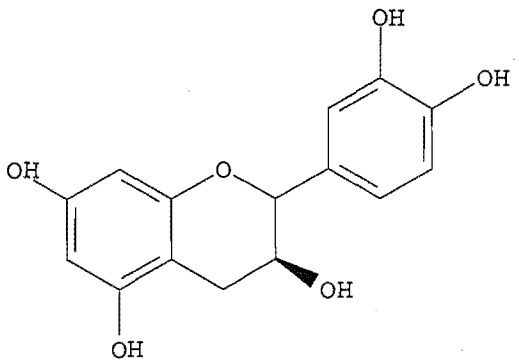
The proanthocyanidins are sometimes referred to as a minor group of flavonoids but this is not true. They are universal in all parts of gymnosperms and are widely distributed in angiosperms except for herbaceous plants (Stafford, 1990). These compounds may be accumulated in very large quantities, up to 30% of the dry weight in some plant parts (Porter, 1988). Like the anthocyanins and flavonols it is thought that these compounds are accumulated mainly in the central vacuoles (Stafford, 1990), although they are also associated with lignin in the cell walls of some species (Swain, 1979; Stafford, 1988). Unlike other classes of flavonoids, the proanthocyanidins and flavan-3-ols are found free rather than glycosylated (Macheix *et al.*, 1990); this is probably due to their greater solubility in an aqueous medium (Stafford, 1990).

The distribution of proanthocyanidins in leaves of the Rosaceae shows a similar pattern to the flavonols with dihydroxyl compounds being dominant (Harborne, 1967). Procyanidin is universal with prodelphinidin limited to a smaller number of species. Propelargonidins have not been reported in the Rosaceae and these are also generally rare, being found only in a few woody angiosperms (Porter, 1988). The flavan-3-ols (+)-catechin and (-)-epicatechin are widespread with (-)-epicatechin generally being dominant (Macheix *et al.*, 1990). (+)-Gallocatechin and (-)-epigallocatechin have also been reported in a small number of species within the Rosaceae. Apples show a proanthocyanidin pattern typical of the Rosaceae, the details of which are discussed in Section Two.

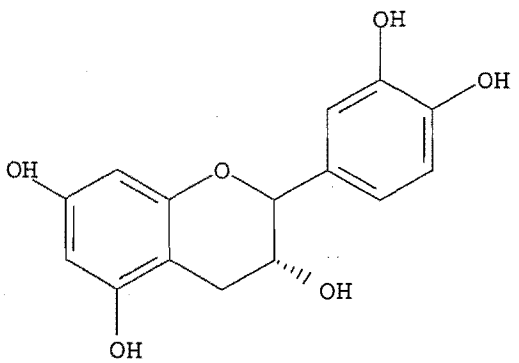
1.2.4 Other flavonoids

A range of other flavonoid pigments are found in plants, the largest group being the flavones along with minor groups including chalcones, aurones, flavanones, isoflavones, biflavonyls and the dihydrochalcones. Most of the flavonoid surveys that mention flavonoid compounds other than those discussed above refer to the leaf flavonoids. The other plant parts, including the fruit, either contain smaller quantities of flavonoids or have not been as well surveyed. The flavonoids present in the fruit are generally more limited in their variation and there are few reports in fruits, other than the anthocyanins, flavonols and proanthocyanidins (Macheix *et al.*, 1990).

Members of the Rosaceae contain both *C*-glycosylflavones and flavone *O*-glycosides. A series of

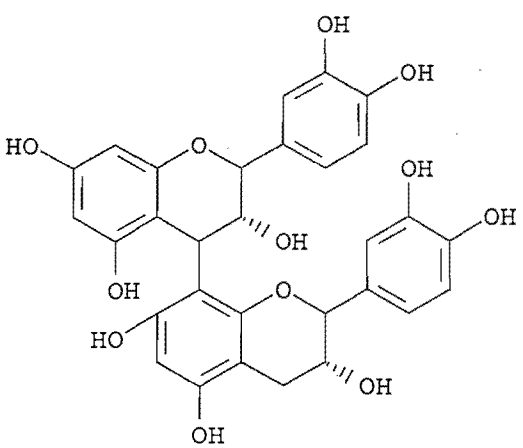


(+)-catechin

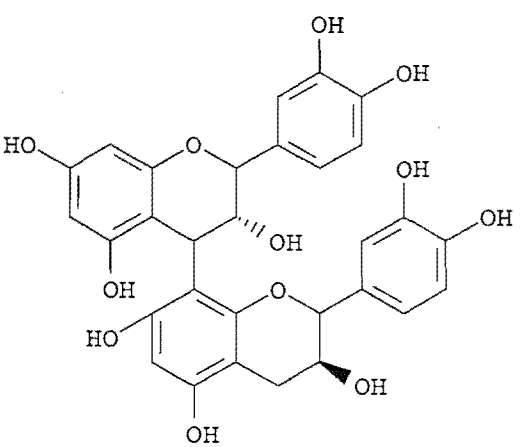


(-)-epicatechin

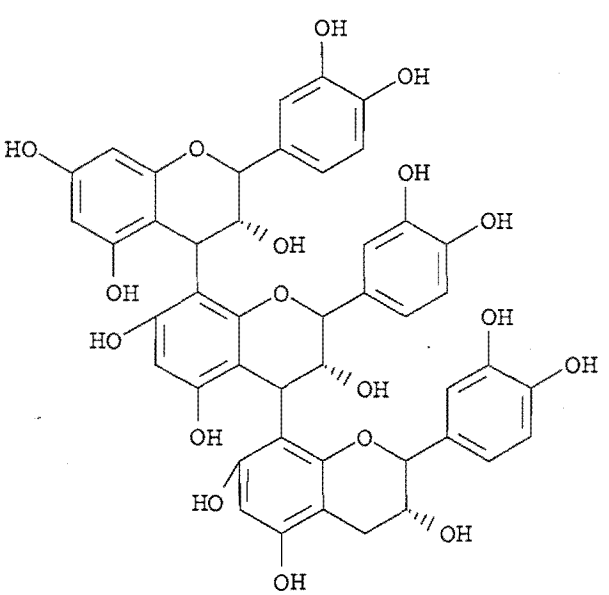
Figure 1.5: Structures of the natural flavan-3-ols, (+)-catechin and (-)-epicatechin



Procyanidin B2, B5



Procyanidin B1



Procyanidin C1

Figure 1.6: Structures of the procyanidins B1, B2 and C1

flavanones are present in a number of species; these include glycosides of naringenin, eriodictyol, sakuranetin (7-*O*-Me naringenin), isosakuranetin (5-*O*-Me naringenin) and pinocembrin (Harborne, 1967). Isoflavones and dihydrochalcones are also present in the family. Work on the subfamily Pomoideae has shown the presence of flavone *O*-glycosides and *C*-glycosylflavones in particular apigenin, luteolin and chrysin glycosides (Challice & Williams, 1968; Challice, 1972 & 1973). A survey of some *Malus* species showed the flavone toringin (chrysin 5-glucoside) and the related dibenzoylmethane glucoside, and the flavanone naringenin 4′-glucoside (Williams, 1982). The dihydrochalcone phloridzin has been reported as present in all *Malus* species examined and in some it occurs together with the related sieboldin and trilobatin (Giannasi, 1988). The distribution of these compounds shows a relationship with the taxonomy of the genus, phloridzin is fairly distinctive of the genus and found in only a few other plant species. The distribution of the dihydrochalcones within *Malus* is of interest as there is a relationship between the flavonoids present and geography within this closely related genus. Western Asian *Malus* species have the dihydrochalcone phloridzin while Eastern Asian species have a glucoside of 3-hydroxyphloretin. Dihydrochalcones are absent from the related *Pyrus* (pear) being replaced in effect by the simple quinol glucoside arbutin (Harborne, 1967). Most of these surveys on other flavonoids have been restricted to leaf and/or bark samples. In apple fruit only the dihydrochalcones have been noted and these were all glycosides of phloretin (Figure 1.7) the details of which are discussed in Section Two.

In summary, the distribution of the various flavonoid classes within the apple (*Malus pumila* Mill.), its genus (*Malus*), the subfamily (Pomoideae) and family (Rosaceae) is shown in Table 1.2. When studying the apple, four groups of flavonoid compounds need to be examined - the anthocyanins, dihydrochalcones, flavonols, and proanthocyanidins including the flavan-3-ols. The flavones and flavanones that are present in many *Malus* species have not been detected in the apple in previous studies and these flavonoids are also absent from the more closely related *Malus* species such as *M. sylvestris*, and *M. micromalis*. However, care must be taken during any study to ensure that the presence of small amounts of these substances is not overlooked.

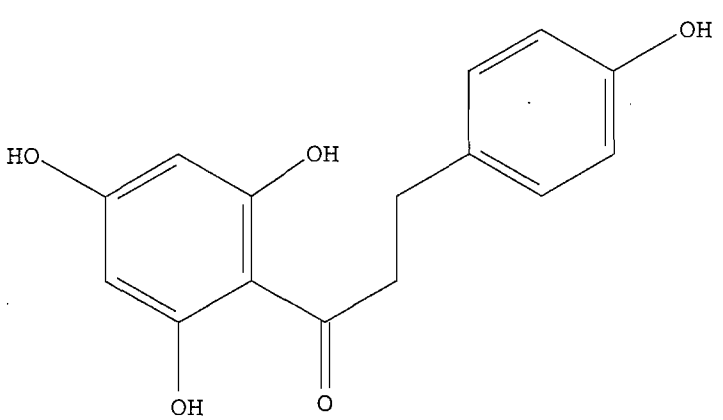


Figure 1.7: Structure of the dihydrochalcone phloretin

Table 1.2: Distribution of the flavonoid classes in the Rosaceae, Pomoideae and *Malus*

Flavonoid Group	Rosaceae	Pomoideae	*Malus*	*Malus pumila*
Anthocyanins	✓	✓	✓	✓
Aurones	-	-	-	-
C-glycosylflavonoids	✓	✓	-	-
Chalcones	✓	-	-	-
Dihydrochalcones	✓	✓[a]	✓	✓
Dihydroflavonols	✓	-	-	-
Flavan-3-ols	✓	✓	✓	✓
Flavan-3,4-diols	-	-	-	-
Flavanones	✓	✓	✓	-
Flavones	✓	✓	✓	-
Flavonols	✓	✓	✓	✓
Isoflavonoids	✓	-	-	-
Proanthocyanidins	✓	✓	✓	✓

✓ Present - Absent

[a] Only present in one genus of the Pomoideae apart from *Malus*

1.3 Apple cultivation and improvement

The apple is among the most ancient of crops known to man, and carbonized fruits from wild trees have been found in prehistoric remains. There are indications of cultivated forms from 6,000 BC and improved forms were clearly being spread from Asia Minor about 2,000 BC (Bellamy & Bollard, 1990). In ancient Greek and Roman literature there are many references to the importance of apples, and descriptions of distinct apple cultivars are found in early Roman writings (Roach, 1985). Improvements in apple cultivars occurred steadily over the years, but in the 19th century efforts became more organized and professional with the recognition of mutations or bud sports (Bellamy & Bollard, 1990). These occur regularly and are usually morphological changes, with increased red colour of the fruit skin being frequently identified (Dermen, 1948 & 1960). In recent years, increased importance has been placed on

breeding programmes brought about mainly through changing preferences for fruit size, colour, texture and taste. Acceptability of our fruit on overseas markets has become the prime determinant of the cultivars grown in New Zealand. In 1990/91 NZ apple exports totalled 213,000 tonnes, of which 50% were cultivars unique to New Zealand in some way (Anon., 1991). However, many other countries are now growing these cultivars and to maintain a competitive edge in the market we must find ways to continue to improve our existing apple cultivars or create new ones.

The colour of an apple's skin is of economic importance since it affects both grading and consumer acceptance. 'Granny Smith' apples may be rejected because of blushing but normally red fruit must be of uniform colour. Consumer research shows that people tend to "buy with their eyes". Colour preferences vary from a green or yellowish green apple to a bright, shiny red fruit and even a mixed colour fruit. Japanese consumers were surveyed as part of the research programme to gain access to Japan for New Zealand apples (Stec & Charles, 1993). Colour was important to them and they preferred bright red, evenly coloured fruit, green colour was disliked as it indicated unripeness and if the red was too dark it suggested that the fruit was over mature. The Japanese have high quality standards and fixed opinions about the ideal attributes of apples destined for their market and New Zealand apple breeders and growers must find ways to meet them.

Plant breeding has resulted in major improvements in many crop plants. However, apple breeding has been laborious and slow and most important commercial cultivars have been chance selections (Bellamy & Bollard, 1990). The essential genetic process in plant breeding is the combining of desirable genetic factors, such as colour, from diverse parents without including less favourable characteristics. However, there are limits to the gene pool of the species and no plant species has, for example, all the genes necessary to give rise to the full spectrum of colours. It is also extremely difficult to change a single factor without influencing other important phenotypic traits and production characteristics (Forkmann, 1991). New opportunities due to advances in molecular genetics and recombinant DNA technologies are now possible and these can overcome some of the barriers of classical breeding procedures. Genes have been isolated from one plant species, cloned into a suitable vector and transferred to another species where they can be expressed and inherited. These techniques allow the manipulation of important traits in a plant, such as colour, to proceed in a more directed fashion (Forkmann, 1991). Thus, it may be possible to introduce a new character, such as even red coloration, directly into a cultivar without changing any of the other existing characteristics such as fruit size, flavour and texture.

The essential background for manipulation of secondary metabolism is an understanding of the biochemistry and molecular biology of the pathway. Ultimately, to manipulate a pathway through genetic engineering, specific genes encoding pathway functions must be cloned and characterized (Gutterson, 1993). As has been discussed above, fruit colour is determined by many different components and factors. There is a complex biochemical pathway that involves a number of different pigments and enzymes that each involve their own gene(s) and control mechanisms (Gutterson, 1993). Because of this there is a need for basic information on the biochemistry before moving on to the molecular biology aspects.

An understanding of specific biochemicals that contribute to a horticultural trait is essential to its modification (Gutterson, 1993). Once the role of the biochemical(s) is determined, the pathway for the biosynthesis and degradation must be elucidated. An understanding of the relative importance of these pathways throughout plant or organ development is also important. Ideally, a flow chart would be constructed with precise information about the rates of each step and the pool size for each intermediate (Figure 1.8). Information on the enzymes catalysing individual reactions of the biochemical pathway is also essential. For apples, some of this ground work has been done, such as the identification of some of the pigments involved, but there are still many gaps in the biochemistry. Little work has been done on the enzymology of the flavonoid pathway with the exception of one enzyme, phenylalanine ammonia-lyase (PAL). However, even this work is of limited application to understanding the formation of colour *in vivo*. How pigment composition relates to the visual appearance of the fruit also needs to be determined, in particular looking at other pigments in addition to the anthocyanins. This project covers some of this biochemistry, thus laying the ground work required for possible future genetic manipulation of fruit colour in apples.

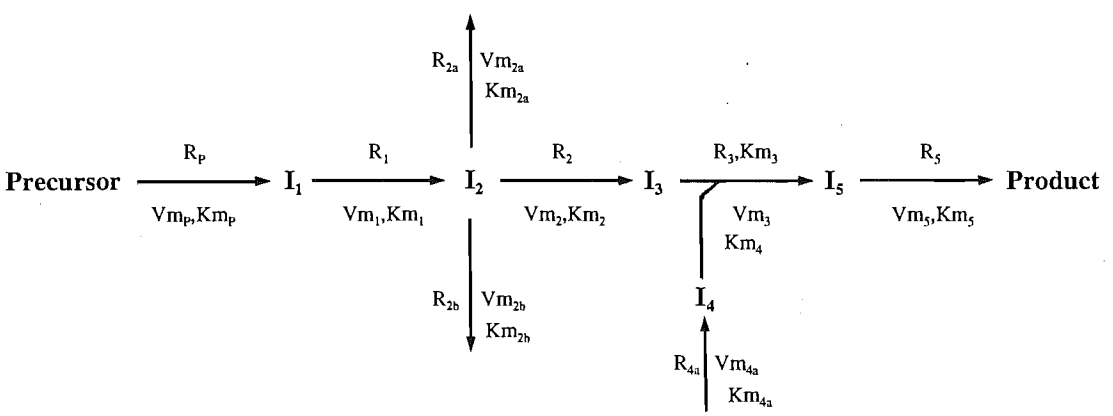


Figure 1.8: Schematic diagram of a hypothetical biosynthetic pathway, indicating information desired for pathway engineering. In addition, information on the concentrations of each intermediate, precursor and product is desired. I=intermediate; R=rate of reaction *in vivo*; Vm=maximal velocity of enzyme catalysing reaction; Km=Michaelis constant of enzyme catalysing reaction (from Gutterson, 1993).

1.4 Biological significance of the flavonoids

When investigating the manipulation of a plant characteristic it is important to consider the function(s) that the compounds involved may play and what effect alteration of these may have on the plant. Phenolics are usually classified as "secondary" metabolites suggesting that they are of secondary importance and such classification has tended to reflect and perpetuate a lack of understanding of their physiological and ecological significance (Swain, 1977). The idea that they are waste products or byproducts has lost favour and a wide variety of biological activities and functions have now been attributed to them (McCallum, 1989). The most important of these include pigmentation. All flavonoids have a high absorbance in the 250 to 270 nm region where proteins and nucleic acids have high absorptive capacity. Flavonols absorb significantly in the range from 330 to 350 nm where NAD^+ and $NADP^+$ cofactors absorb strongly, and anthocyanins absorb strongly in the range from 520 to 560 nm where the mammalian eye has its highest sensitivity and where plant photoreceptors such as chlorophyll, carotenoids and phytochrome have minimal absorptivity (McClure, 1982). It is probably in the area of visual perception and light absorbance that the strongest case can be made for unambiguous functions of flavonoids (Harborne, 1972). The red colour probably has some role as an attractant for fruit, and hence seed dispersal.

A wide range of other functions has been attributed to the flavonoids and functions include antioxidant properties, enzyme inhibition, precursors of toxic substances, promotion and inhibition of plant growth hormones, morphogenesis and sex determination, and various pharmacological and clinical properties (McClure, 1982). The high concentrations of flavonoids in fruit skins also suggests that they may have a role as defence chemicals against pathogens and predators (Luckner, 1990).

With specific regard to apples, a number of different functions have been reported. Flavonoids may have a role in superficial scald resistance (Albrigo & Childers, 1970). Duvenage and de Swardt (1973) also reported on the relationship between proanthocyanidin content of apple peel and the degree of browning or susceptibility to superficial scald. Naturally occurring flavonols of apples have been shown to regulate fruit softening at room and storage temperature (Lidster *et al.*, 1986). Flavonoids may also have a role in scab resistance as high levels of flavan-3-ols have been reported to confer resistance, possibly by inhibiting the associated fungal infection (Treutter & Feucht, 1990). The phenolic compounds of apples are also involved in enzymic browning which is a major problem for the processing industry (Walker, 1962 & 1963; Oleszek *et al.*, 1988; Burda *et al.*, 1990).

1.5 Experimental strategy and outline

There are four major questions to answer in attempts to understand the biochemistry of apple fruit colour:

(1) What pigments are present in apple fruit skin?

(2) How does pigment composition relate to fruit colour - what are the differences between genotypes and species?

(3) What changes occur during fruit development - are changes qualitative and/or quantitative?

(4) What are the details of the biosynthetic pathway for the major pigment group?

For the purpose of this thesis, the work has been divided into four sections that each reflect an emphasis on one of these questions. In each section the studies have been guided by the key question:

"What are the important factors in determining apple colour?"

In the first section the flavonoid pigments present in apples were examined are where possible identified to determine the chemical nature and the relationship between the compounds. There have been a number of studies on the flavonoid groups present in apples but in some cases there are conflicting results and no detailed study has been made of genotypes originating in New Zealand. Improvements to the existing HPLC methodology were also investigated to develop a single method of identification and quantification of all the flavonoids present in apple skin.

Quantification of the pigment groups is the theme of the second section. The three groups of pigments (carotenoids, chlorophyll and flavonoids) were measured in a wide range of different coloured apple genotypes and species. Most previous studies have been done on a single group of compounds and have not been integrated to compare the overall effects of pigment composition on fruit appearance. Thus, attempts were made to relate the composition and concentrations of these pigments to the range of colours seen. Some of the other factors that have an influence on fruit colour were also investigated.

The third section studied the changes that took place in the concentrations of the different pigment groups during the development of the fruit and investigated whether there were changes in individual flavonoids. Red, yellow and green apple cultivars were examined to compare differences in the flavonoid groups and these differences were related to control of flavonoid biosynthesis.

The biosynthesis of the flavonoids was investigated further in the fourth section. There have been very few previous studies of the enzymes of flavonoid biosynthesis in apple fruit and these have largely been limited to studies on PAL. The aim of this study was to expand on this to understand how flavonoid biosynthesis was controlled in apples. Comparisons were made between red and non-red cultivars to determine which point(s) in the pathway were critical for reddening. Changes in three enzymes of the flavonoid pathway were examined and related to changes in flavonoid levels and attempts were made to determine the rate-limiting step in the pathway. Apples with chimeral stripes and UV induction of reddening were also studied to provide more information on the regulation of reddening.

Section 2 - IDENTIFICATION OF THE FLAVONOIDS IN APPLE SKIN

2.1 Introduction

2.1.1 Anthocyanins

The anthocyanin responsible for the red colour of apple skin was tentatively identified as long ago as 1931 by Robinson and Robinson as a 3-monoside of cyanidin. Then Duncan & Dustman (1936) and Sando (1937) both identified the predominant anthocyanin pigment in apples as cyanidin-3-galactoside, commonly named idaein (Figure 2.1). Since then, a number of workers have confirmed this as the major pigment in a number of cultivars and *Malus* species from around the world (Sun & Francis, 1967; Pais & Gombkoto, 1967; Durkee & Jones, 1969). Harborne (1967) surveyed 15 *Malus* species and found that they all contained cyanidin-3-galactoside, and in two species a cyanidin-3-pentoside was also identified. However, Samorodova-Bianki & Bazarova (1970) mentioned only one pigment, purported to be cyanidin arabinosylgalactoside, from the skin and flesh of 'Komsomolets' apples. This has not been reported elsewhere and is either specific to this cultivar, having arisen from a mutation, or is a mis-identification.

There have also been several reports on the composition of the minor anthocyanins present in different apple cultivars. These are all cyanidin derivatives but of varying glycoside composition (Table 2.1). Cyanidin-3-gentiobioside has been reported by Kolesnik & Putintseva (1963) but it has not been reported elsewhere. Sun & Francis (1967) reported two minor pigments, which they identified as cyanidin-3-arabinoside and cyanidin-7-arabinoside, as well as confirming the identity of cyanidin-3-galactoside as the main pigment. They surveyed 83 cultivars, and of these 74 contained all three

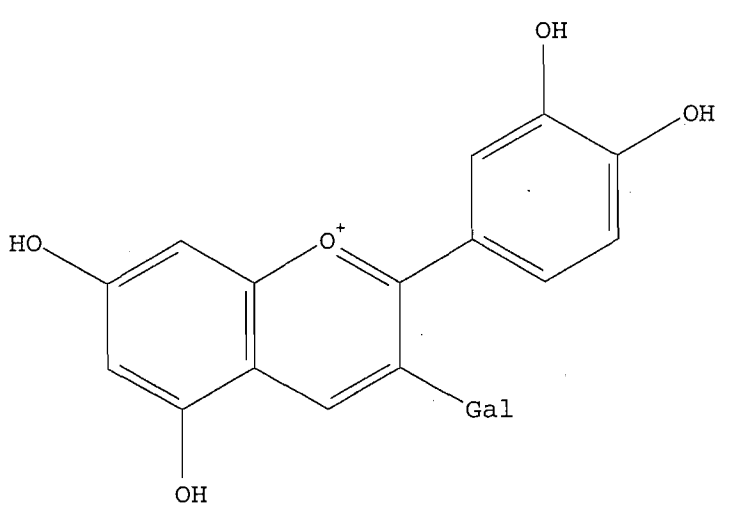


Figure 2.1: Structure of cyanidin-3-galactoside

Table 2.1: Summary of some previously reported anthocyanins and their levels in apple skin

Anthocyanin	Quantity (mg g^{-1} FW)	Apple cultivar	Reference
cyanidin-3-arabinoside	0.02[a]	'Scugog'	Mazza & Velioglu (1992)
	-	'Jonathan' &	Posokhlyarova (1976)
	-	'Wagner'	
	-	'Red Delicious'	Sun & Francis (1967)
	-	various[b]	Timberlake & Bridle (1971)
cyanidin-3-arabinoside (acylated)	-	various[b]	Timberlake & Bridle (1971)
cyanidin arabinosyl-galactoside	0.27	'Komsomolets'	Samorodova-Bianki & Bazarova (1970)
cyanidin-7-arabinoside	-	'Jonathan' &	Posokhlyarova (1976)
	-	'Wagner'	
	-	'Red Delicious'	Sun & Francis (1967)
cyanidin-3-galactoside	-	'Winesap'	Duncan & Dustman (1936)
	0.44	'Cox's Orange'	Knee (1972)
	0.04[a]	'Scugog'	Mazza & Velioglu (1992)
	-	'Jonathan' &	Posokhlyarova (1976)
	-	'Wagner'	
	-	'Jonathan' &	Sando (1937)
	-	'Stayman Winesap'	
	-	'Red Delicious'	Sun & Francis (1967)
cyanidin-3-galactoside (acylated)	-	'Jonathan'	Pais & Gombkoto (1967)
	-	various[b]	Timberlake & Bridle (1971)
cyanidin-3-gentiobioside	-	?	Kolesnik & Putintseva (1963)
cyanidin-3-glucoside	0.03[a]	'Scugog'	Mazza & Velioglu (1992)
	-	various[b]	Timberlake & Bridle (1971)
	-		
cyanidin-3-glucoside (acylated)	-	'Jonathan'[c]	Pais & Gombkoto (1967)
	-	various[b]	Timberlake & Bridle (1971)
cyanidin-3-xyloside	0.01[a]	'Scugog'	Mazza & Velioglu (1992)
	-	various[b]	Timberlake & Bridle (1971)
cyanidin-3-xyloside (acylated)	-	various[b]	Timberlake & Bridle (1971)
total anthocyanin	0.43-0.82	'Waldhofler'	van Buren *et al.* (1966)
	0.1-21.6	N.Z. cultivars	Walker (1964)

- not measured
[a] concentration in combined skin and flesh
[b] 'Red Delicious', 'Stoke Red', 'Jonathan', 'Tremlett's Bitter', 'Ingrid Marie' and 'Cox's Orange Pippin'
[c] two forms

pigments, six had only two, two cultivars contained only the major anthocyanin and one cultivar contained no anthocyanins. These three anthocyanins were also reported from 'Jonathan' and 'Wagner' apples grown in the USSR (Posokhlyarova, 1976). Three minor pigments were noted by Pais & Gombkoto (1967) in 'Jonathan' apples, these were an acylated cyanidin-3-galactoside and two forms of an acylated cyanidin-3-glucoside. Durkee & Jones (1969) noted two minor pigments but they were not identified. Because of these conflicting reports a careful examination was carried out on a number of apple cultivars by Timberlake & Bridle (1971). The minor pigments were identified as cyanidin-3-glucoside, 3-arabinoside, and 3-xyloside as well as acylated derivatives of all four glycosides. However, the composition varied according to the methods of extraction and purification. More recently, the anthocyanins in the skin and flesh of red-flesh apples ('Scugog', *Malus pumila* var. *niedzewetzkyana*) were separated by HPLC and identified (Mazza & Velioglu, 1992). The predominant anthocyanin was cyanidin-3-galactoside with cyanidin-3-glucoside, 3-arabinoside, and 3-xyloside also present although in lower concentrations. They did not detect the presence of the acylated compounds that have been reported previously (Pais & Gombkoto, 1967; Timberlake & Bridle, 1971).

The only study on the anthocyanins in New Zealand apple cultivars has been done by Walker (1964). Cyanidin-3-galactoside was confirmed as the major anthocyanin present but no mention was made of any minor components. Thus there was a need to survey New Zealand-grown cultivars to determine what was present since previous reports of minor components are varied. The levels of these anthocyanins were also determined to use for later comparisons with fruit colour and copigmentation studies.

In addition to qualitative differences, quantitative differences have also been reported. Large variations have been noted in the concentrations of the anthocyanins, ranging from some varieties that contain none up to a high of 21.6 mg g^{-1} FW in dark red 'Democrat' apples (Walker, 1964). There is also variation in the levels reported for a single cultivar, for example 'Cox's Orange' where Knee (1972) reported average concentrations of 0.44 mg g^{-1} FW yet Walker (1964) reported levels from 0.41 to 2.96 mg g^{-1} FW. There is also variation in the percentage composition of the major anthocyanin and minor components. Sun & Francis (1967) reported that cyanidin 3-galactoside accounted for about 68% of the total anthocyanins with the 3-arabinoside making up 24% and the 7-arabinoside 8%. In the cultivars analysed by Timberlake & Bridle (1971), cyanidin 3-galactoside accounted for 83 to 94%, 3-arabinoside for 4 to 13%, 3-glucoside for 1 to 8%, and 3-xyloside for less than 1% of the total anthocyanins. Although the same compounds were reported by Mazza & Velioglu (1992), the composition was significantly different (3-galactoside 39%, 3-glucoside 27%, 3-arabinoside 23% and 3-xyloside 11%). The reasons for these variations have not been determined.

There seems to be some variation in the composition of these minor components, both qualitatively and quantitatively. This may be due to environmental factors and cultural practices and where there is variation within a cultivar this could be due to mutants or sports. These factors have been discussed, with specific regard to apples, by a number of workers (Walter, 1967; Saure, 1990; Mazza & Miniati, 1993). However, without a uniform method for the extraction, identification and quantification it is very difficult

to compare the results from different reports. One minor component that has been the subject of some debate is the presence of the 7-arabinoside initially reported by Sun & Francis (1967) and also noted by Posokhlyarova (1976). Where arabinose occurs in trace or small amounts it may be a contaminant. Arabinose is produced as an artifact, by the action of mineral acid (usually hydrochloric acid) in the solvent system on the paper during paper chromatography (Timberlake *et al.*, 1971). Paper-derived arabinose then interferes with the normal detection of the sugars and gives misleading results. This could possibly explain the report of arabinosylgalactoside by Samorodova-Bianki & Bazarova (1970) in their study of anthocyanins from 'Komsomolets' apples. The identification of the 7-arabinoside was made on the basis of chromatographic and spectral data from component **III** of an extract of American 'Red Delicious' apples (Sun & Francis, 1967). However, Timberlake & Bridle (1971) later found that in component **III**, from English and French fruit of the same cultivar, all of the sugars were attached in the '3' position and that the high R_f was due to the presence of anthocyanins with acyl groups (of unknown nature). The high ratio of E_{275}/E_{max} that was reported for cyanidin-7-arabinoside may be due to associated procyanidins that were difficult to separate chromatographically from the anthocyanins (Timberlake, 1981). The production of arabinose can be avoided by using acetic acid instead of mineral acid during extraction and purification (Timberlake *et al.*, 1971). This use of milder acid in the extraction is also beneficial if acylated compounds are present (Harborne, 1986). Since acylated compounds have been reported by some researchers (Pais & Gombkoto, 1967; Timberlake & Bridle, 1971) this procedure may help to determine if they are present or not. For these reasons acetic acid was used in all extraction and purification procedures as opposed to the hydrochloric acid used by many previous researchers.

2.1.2 Flavonols

The first flavonol identified from apple skin was the aglycone quercetin (Sando, 1924), but it was not until some years later that identification of the first glycoside was reported. Quercetin-3-galactoside (Figure 2.2) was identified as the major component from 'Grimes Golden' and 'Jonathan' apples (Sando, 1937). Since then a range of quercetin glycosides have been detected and identified from the skin of a number of apple cultivars (Table 2.2). The most extensive range of glycosides, relating to a single aglycone, in a single species, was reported from the skin of 'Grimes Golden' apples (Siegelman, 1955). These were identified as quercetin-3-galactoside (hyperin), quercetin-3-glucoside (isoquercetrin), quercetin-3-arabinoside (avicularin), quercetin-3-rhamnoside (quercitrin), quercetin-3-rutinoside (rutin) and quercetin-3-xyloside (reynoutrin). Hoester-Auer (1964) identified three quercetin glycosides and also reported the presence of free quercetin. Subsequent workers have not detected this so it may have arisen as a result of the extraction methods employed. Siegelman (1955) noted quercetin in some extracts but concluded that it arose as a hydrolysis product in the extraction procedure. Fisher (1966) also reported these same six compounds from 'Democrat' fruit and in addition a quercetin diglucoside (meratin) was detected, but this has not been noted by any other workers. Dick *et al.* (1987) reported five quercetin glycosides (3-

Table 2.2: Summary of some previously reported flavonols and their levels in apple skin

Flavonol glycoside	Quantity (mg g^{-1} FW)	Apple cultivar	Reference
kaempferol glycosides	0.007	'Cox's Orange'	Herrmann (1976)
	0.002	'Gravenstein'	Herrmann (1976)
quercetin-3-arabinoside	0.13-0.2	various[a]	Burda *et al.* (1990)
	-	'Spartan'	Dick *et al.* (1987)
	0.5-0.8	various[b]	McRae *et al.* (1990)
	-	'Rhode Is. Greening'	Oleszek *et al.* (1988)
quercetin-3-diglucoside	-	'Democrat'	Fisher (1966)
quercetin-3-galactoside	0.22-0.37	various[a]	Burda *et al.* (1990)
	-	'Spartan'	Dick *et al.* (1987)
	-	'Rhode Is. Greening'	Oleszek *et al.* (1988)
	-	'Grimes Golden' &	Sando (1937)
	-	'Jonathan'	
	-	'Grimes Golden'	Siegelman (1955)
quercetin-3-galactoside + glucoside	0.55-0.92	various[b]	McRae *et al.* (1990)
quercetin-3-glucoside	0.07-0.13	various[a]	Burda *et al.* (1990)
	-	'Spartan'	Dick *et al.* (1987)
	-	'Rhode Is. Greening'	Oleszek *et al.* (1988)
	-	'Grimes Golden'	Siegelman (1955)
quercetin-3- rhamnoside	0.20-0.23	various[a]	Burda *et al.* (1990)
	-	'Spartan'	Dick *et al.* (1987)
	0.07-0.66	various[b]	McRae *et al.* (1990)
	-	'Rhode Is. Greening'	Oleszek *et al.* (1988)
	-	'Grimes Golden'	Siegelman (1955)
quercetin-3-rutinoside	0.06-0.19	various[b]	McRae *et al.* (1990)
	-	'Grimes Golden'	Siegelman (1955)
quercetin-3-xyloside	0.10-0.15	various[a]	Burda *et al.* (1990)
	-	'Spartan'	Dick *et al.* (1987)
	0.12-0.30	various[b]	McRae *et al.* (1990)
	-	'Rhode Is. Greening'	Oleszek *et al.* (1988)
	-	'Grimes Golden'	Siegelman (1955)
total quercetin	0.41-18.8	N.Z. cultivars	Walker (1964)
	0.56-33.3	'Golden Delicious'	Workman (1963)
	0.60-17.16	'Grimes Golden'	Workman (1963)

- not measured

[a] 'Golden Delicious', 'Empire', 'Rhode Island Greening'

[b] 'Jerseymac', 'Gravenstein', 'Northern Spy', 'McIntosh', 'Golden Delicious', 'Spartan', 'Cortland', 'Red Delicious'

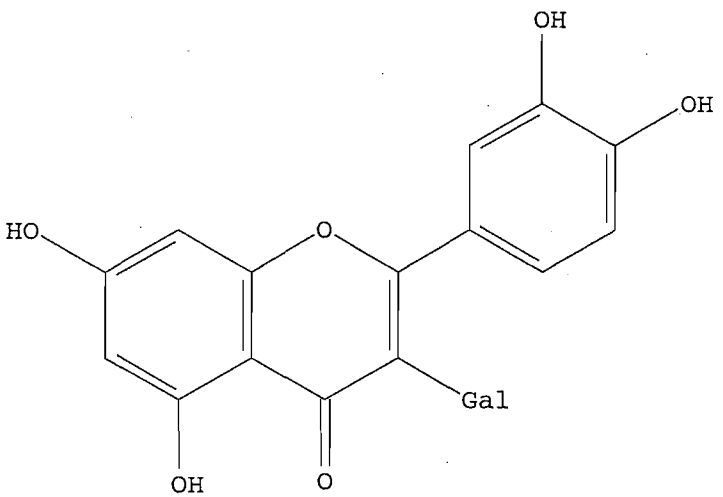


Figure 2.2: Structure of quercetin-3-galactoside

arabinoside, 3-galactoside, 3-glucoside, 3-rhamnoside and 3-xyloside) from 'Spartan' apples. These same compounds were noted in 'Rhode Island Greening' apples by Oleszek *et al.* (1988) and Burda *et al.* (1990) detected them in 'Golden Delicious' and 'Rhode Island Greening' cultivars. Quercetin 3-galactoside has generally been reported as the predominant glycoside although McRae *et al.* (1990) reported quercetin-3-rhamnoside as the major compound in a number of cultivars. They also reported that the other pigments were the same as those found previously. Kaempferol glycosides have also been reported in apple skins but at much lower concentrations (van Buren, 1970; Herrmann, 1976). The identity of the specific glycosides present has not been reported. However, some workers have noted that kaempferol glycosides are absent (Macheix *et al.*, 1990). It is unclear whether these differences were due to cultivar variation or that kaempferol glycosides were present at a much lower concentration than the quercetin glycosides and so may not have been detected by some workers.

Although the identification of the flavonols present in apple skins has been relatively well researched there are still some areas requiring investigation. As with the anthocyanins, there has been a great deal of variation in the concentrations of the flavonols reported for apple skin. Study of a large number of cultivars may help determine whether these differences are cultivar-specific or due to environmental influences. For New Zealand apples, the only major study has been done by Walker (1964), whose work confirmed the major flavonol pigment as quercetin-3-galactoside in a number of cultivars. He did not report on the presence or absence of other flavonol pigments in these apple cultivars. As for the anthocyanins, the composition and concentration of the quercetin glycosides was measured for a number of New Zealand-grown apple genotypes and species. This data also provided information for the study of copigmentation which is discussed in Section Three.

The presence of some minor compounds has been noted by some workers but these have not been

identified mainly due to the low concentrations (Dick *et al.*, 1987). The identification of these compounds was made where possible. Confirmation of the presence or absence of kaempferol glycosides in apple skins was also made and where detected these glycosides were tentatively identified. The concentration(s) of these glycosides were also measured to determine whether they had an effect on colour through copigmentation with the anthocyanins. Since kaempferol has a different hydroxylation pattern from quercetin it may have a different copigmentation effect if present in significant amounts.

Flavonols are generally easier to study than the anthocyanins since they are more stable (Harborne, 1967). Most of the early studies were limited because they were based on the techniques of thin-layer chromatography (TLC) and column chromatography. Separation and identification of these glycosides by conventional chromatographic methods is very difficult due to the similarity of their molecular weight and polarity (de Bernardi *et al.*, 1984). This is particularly so for apples since the flavonols present are similar, being largely quercetin glycosides. The other main problem in studying apple flavonoids is that some of the minor flavonols are present in low concentrations (Macheix *et al.*, 1990). The development of HPLC methods for the separation and detection of the flavonols has made the task much simpler and more reliable.

2.1.3 Proanthocyanidins

Most of the work done on the proanthocyanidins in apples has been the characterisation of these compounds in ciders where they contribute to its bitterness and astringency (Lea & Timberlake, 1974). The main proanthocyanidin was identified as procyanidin B2 (an epicatechin dimer) by Lea & Timberlake (1974) whilst procyanidin B1 (epicatechin/catechin dimer), procyanidin B5 (epicatechin dimer) and a procyanidin trimer, possibly C-1 (epicatechin trimer), were minor components. In addition to these, Wilson (1981) reported other trimers of similar composition present in small quantities. A similar composition would probably be expected for the apple skin. Although proanthocyanidins have been noted in apple skins, there are fewer reports on the actual identity and concentration of these compounds compared to the other flavonoids (Table 2.3). Four distinct proanthocyanidins in apples were reported by Williams (1960), these all yielded cyanidin on acid hydrolysis and one also gave (-)-epicatechin; further identification of these compounds was not reported. Ito & Joslyn (1965) also described a number of proanthocyanidins (misnamed leucoanthocyanidins) that were highly polymerized and complex. On hydrolysis, these compounds yielded cyanidin, pelargonidin, (-)-epicatechin and (+)-catechin. In a study on 'Waldhöfler' apples, van Buren *et al.* (1966) reported one proanthocyanidin that was a dimer, or possibly an oligomer, and that contained (-)-epicatechin and 5,7,3′,4′ flavan-3,4-diol as constituents. Other proanthocyanidins yielded cyanidin, (-)-epicatechin and a small amount of pelargonidin on hydrolysis. Although cyanidin is an expected component, since it is usually present in apples, it is unusual that pelargonidin is formed since apples contain few flavonoids with this hydroxylation pattern (4′-OH).

Table 2.3: Summary of some previously reported proanthocyanidins and their levels in apple skin

Proanthocyanidin	Quantity (mg g^{-1} FW)	Apple cultivar	Reference
(+)-catechin	-	'Schöner von Boskoop'	Mosel & Herrmann
	-	& 'Golden Delicious'	(1974a & b)
	0.17-4.3	various[a]	Treutter & Feucht (1990)
	0.12	'Waldhöfler'	van Buren *et al.* (1966)
(-)-epicatechin	0.13-0.67	various[b]	Burda *et al.* (1990)
	-	'Schöner von Boskoop'	Mosel & Herrmann
	-	& 'Golden Delicious'	(1974a & b)
	0.74-19.5	various[c]	Prabha & Patwardhen (1985a)
	2.2-4.1	various[a]	Treutter & Feucht (1990)
	0.25	'Waldhöfler'	van Buren *et al.* (1966)
(-)-epigallocatechin	0.006	'Jonathan'	Mosel & Herrmann
	0.002	'Schöner von Boskoop'	(1974a & b)
(+)-gallocatechin	-	'Schöner von Boskoop'	Mosel & Herrmann
	-	& 'Golden Delicious'	(1974a & b)
	trace-0.045	various[c]	Prabha & Patwardhen (1985a)
procyanidin B2	0.12-0.60	various[b]	Burda *et al.* (1990)
	0.43-1.35	various[a]	Treutter & Feucht (1990)
total flavan-3-ols	0.45-14.31	various[d]	McRae *et al.* (1990)

- not measured
[a] 'Golden Delicious', 'Jonagold', 'Gala', 'Royal Gala', 'Elstar', 'Prima', 'Coop'
[b] 'Golden Delicious', 'Empire', 'Rhode Island Greening'
[c] 'Aumburi', 'Royal Delicious', 'Kesari', 'Red Delicious', 'Rich-a-Red'
[d] 'Jerseymac', 'Gravenstein', 'Northern Spy', 'McIntosh', 'Golden Delicious', 'Spartan', 'Cortland', 'Red Delicious'

The monomeric flavan-3-ols, (+)-catechin and (-)-epicatechin, are commonly reported with (-)-epicatechin being present in much higher concentrations (van Buren *et al.*, 1966). There have also been several reports of the presence of small amounts of (+)-gallocatechin in apple skin (Mosel & Herrmann, 1974a & b; Prabha & Patwardhen, 1985a). The related (-)-epigallocatechin has also been identified in trace amounts from two apple cultivars (Mosel & Herrmann, 1974a & b) but has not been reported elsewhere. More recently the phenolic compounds in apples were determined by HPLC (Burda *et al.*, 1990) and (-)-epicatechin and procyanidin B_2 were reported as the main components in the skin of three apple cultivars ('Golden Delicious', 'Empire' and 'Rhode Island Greening').

There are no reports of monomeric flavan-3,4-diols (leucoanthocyanidins) in apple skins, which is not surprising since they are rare in fruit (Macheix *et al.*, 1990). The proanthocyanidins present in New Zealand-grown apple cultivars have not been determined. As with the anthocyanins and flavonols, the composition and levels of the proanthocyanidins and the related flavan-3-ols were determined for a number

of genotypes and species. In particular the presence or absence of (+)-gallocatechin and (-)-epigallocatechin was be noted since the presence of these compounds is debated. Any other minor compounds that may be present in the skin such as those reported in cider, for example procyanidin B5, were looked for.

2.1.4 Other flavonoids

As was mentioned earlier, the only other class of flavonoids previously detected in apples has been the dihydrochalcones (Table 2.4). Phloretin 2′-*O*-glucoside (Figure 2.5), more commonly named phloridzin, has long been known to be present in some apple tissues and in apple juice (Lea & Timberlake, 1974). For many years there was debate over its presence in the skin of the fruit. However, more recent data from HPLC and characterisation by ^{1}H and ^{13}C-NMR has confirmed its presence in apple peel (Dick *et al.*, 1987). Since then a number of workers have detected and quantified dihydrochalcone glycosides in apple fruit skin. Burda *et al.* (1990) reported the presence of two dihydrochalcones, phloretin xylogalactoside and phloridzin, in three apple cultivars and these were present in the flesh and the skin of the fruit, unlike the flavonols which were only present in the skin (Workman, 1963). Oleszek *et al.* (1988) also reported the presence of phloridzin but they identified phloretin xyloglucoside not xylogalactoside in the skin of 'Rhode Island Greening' apples.

There have been no reports on the presence or absence of the dihydrochalcones, or any other classes of flavonoids, in New Zealand-grown apple cultivars. The identification and quantification of any

Table 2.4: Summary of some previously reported dihydrochalcones and their levels in apple skin

Dihydrochalcone	Quantity (mg g^{-1} FW)	Apple cultivar	Reference
phloretin glucoside (phloridzin)	0.10-0.15	various[a]	Burda *et al.* (1990)
	0.004-0.02	various[b]	Coseteng & Lee (1987)
	0.09-0.33	various[c]	Dick (1986)
	-	'Spartan'	Dick *et al.* (1987)
	-	'Rhode Island Greening'	Oleszek *et al.* (1988)
phloretin xylogalactoside	0.06-0.23	various[a]	Burda *et al.* (1990)
phloretin xyloglucoside	-	'Rhode Island Greening'	Oleszek *et al.* (1988)

- not measured
[a] 'Golden Delicious', 'Empire', 'Rhode Island Greening'
[b] 'Empire', 'Cortland', 'McIntosh', 'Golden Delicious', 'Rome', 'Rhode Island Greening', 'Classic Delicious'
[c] 'Jersey Mac', 'Gravenstein', 'McIntosh', 'Cortland', 'Spartan', 'Golden Delicious', 'Red Delicious', 'Northern Spy'

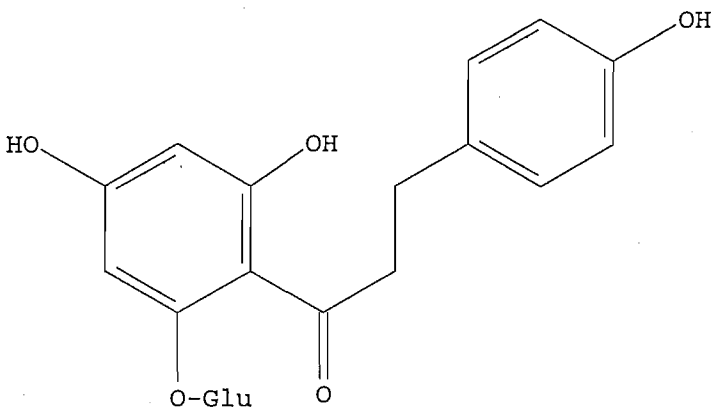


Figure 2.3: Structure of phloridzin (phloretin-2′-glucoside)

dihydrochalcones detected in the genotypes or species examined was undertaken. The presence of other flavonoid compounds was not excluded, although if these compounds were detected they would probably be present in only trace amounts which would make identification difficult. Based on the types of flavonoids found in other *Malus* species, only the flavones (apigenin and luteolin glycosides) and flavanones (naringenin and eriodictyol glycosides) are expected.

2.1.5 Distribution of the flavonoids

In some plants, there are similarities between the flavonoids found in different plant parts and in others major differences. Troncet (1972) studied the patterns of flavonoids in the epidermal peels, seeds and pulp of 70 fruits. In all cases the superficial layer had the same flavonoids as the epidermis of other aerial parts of the plant. Flavonoids were not present in the flesh but usually occurred in the seeds. The accumulation of phenolics in fruits may be higher or lower than other parts of the plant such as the bark or leaves (van Buren, 1970). Fruits and flowers often contain some flavonoid types, such as the anthocyanins, whilst the other parts of the plant have very little or none.

Anthocyanins are most frequently located in the external tissue (epidermis and a few subepidermal layers) but occasionally they may be distributed through the whole fruit such as 'Scugog' apples (Mazza & Velioglu, 1992). They are also sometimes found in the bark and leaves of the apple tree. The composition of the anthocyanins in the bark is the same as has been reported for the fruit with cyanidin-3-galactoside, 3-arabinoside and 7-arabinoside (Mazza & Miniati, 1993). These anthocyanins are located in the hypodermal cells and are at a higher concentration in winter than summer. Anthocyanins may occur in the leaves particularly under stress conditions such as water-logging (Andersen *et al.*, 1984).

In the apple phloridzin is present in significant amounts in the other organs but is present in much lower concentrations in the fruit (van Buren, 1970). It has been reported as the principal phenolic of apple leaf and bark and some also occurs in the seeds (Williams, 1960). This author also reported the presence of phloretin-3-xyloglucoside but only in the bark. As mentioned above, there have been various reports of its presence and absence in the fruit, being located in the flesh as well as the skin (Burda *et al.*, 1990). The flavonols, quercetin and kaempferol glycosides, have been reported from several parts of the tree (bark, fruit and leaf). The quercetin glycosides were predominant in all cases (Williams, 1960).

The distribution of the various other flavonoids within the fruit has been reported by a number of workers. Total flavonoids are reported as being much higher in the skin compared to the flesh (van Buren, 1970; Prabha & Patwardhan, 1985b). Natural flavan-3-ols, (+)-catechin and (-)-epicatechin, were reported as present in the skin at three times the concentration of the flesh (Sal'kova & Bekbulatova, 1965). Prabha & Patwardhan (1985b) noted that total proanthocyanidins were seven to twenty times higher in the skin than the flesh. The flavonols have been reported as totally absent from the flesh by some workers (Workman, 1963; Burda *et al.*, 1990) yet other workers reported their presence, although at much lower concentrations (Herrmann, 1976). Proanthocyanidins have been reported only in the fruit but their presence has not been excluded from other plant parts. The composition of the flavonoids in different apple tissues was examined in order to relate distribution to control of flavonoid biosynthesis.

2.1.6 HPLC

Early studies of the flavonoids were limited to qualitative analysis based on the techniques of thin-layer chromatography (TLC) or column chromatography (Oleszek *et al.*, 1988). However, since 1973 there has been a dramatic increase in interest in the application of HPLC to study flavonoids. Two general reviews on the use of HPLC in flavonoid analysis have been written by Daigle and Conkerton (1983 & 1988). HPLC has a number of advantages over many other methods of analysis: (1) short analysis time, (2) high resolution, (3) no derivatisation required, (4) no risk of thermal decomposition and, (5) easy quantification.

Reversed phase (RP) C_{18} columns are the most commonly used for flavonoid analysis with methanol/water or acetonitrile/water the preferred solvent systems. HPLC has been applied to a wide range of plant tissues including apples and apple juice. Wilson (1981) reported the HPLC separation of apple juice phenolic compounds, these included (+)-catechin, (-)-epicatechin, chlorogenic acid, phloridzin, phloretin xyloglucoside and various polymeric procyanidins. These analyses were carried out on either a cyano (CN) column run in the normal phase using an acidified tetrahydrofuran (THF)-hexane solvent gradient, or a C_{18} column using methanol-aqueous KH_2PO_4 solvent gradient. Flavonoid glycosides of 'Spartan' apple peel were analyzed by Dick *et al.* (1987) by RP-HPLC using a THF and trifluoroacetic (TFA) acid solvent system. Quercetin-3-glucoside and 3-galactoside were not resolved in this system but resolution of all the other main flavonoid glycosides was achieved although there was some overlap of

quercetin-3-arabinoside and 3-xyloside. Their method did not preclude the occurrence of other minor compounds since they identified only the five main peaks. However, their extraction methods did not yield flavonoid disaccharides such as rutin and they did not examine the anthocyanins or proanthocyanidins. Oleszek *et al.* (1988) reported the separation of five quercetin glycosides and two phloretin glycosides from apple skin plus (+)-catechin, (-)-epicatechin and procyanidins. They used a micropreparative RP C_{18} column with an acetonitrile/acetic acid isocratic elution. More recently the anthocyanins in red-fleshed apples have been separated by RP-HPLC using formic acid/methanol. Separation of four anthocyanin glycosides was achieved (Mazza & Velioglu, 1992). A number of other phenolic compounds were also separated in this system and included three quercetin glycosides, two phenolic acids, two flavan-3-ols and one dihydrochalcone glycoside.

Improvements are still needed to existing HPLC methodology to separate all of the components from apple fruit skins. Such separation would allow quantification of the individual flavonoid compounds from a single sample. The methods outlined above were used as a starting point to develop method(s) for the separation and quantification of all those flavonoids present in apples, particularly the quercetin glycosides.

2.2 Materials and methods

Materials

Flavonoid standards

Cyanidin, cyanidin-3-galactoside, cyanidin-3-glucoside, delphinidin, malvidin, pelargonidin, peonidin, petunidin, quercetin-3-galactose, quercetin-3-glucose, quercetin-3-rhamnose and quercetin-3-xylose were obtained from Plantech U.K. (Building L-11, The University, London Road, Reading RG 15AQ, England).

(+)-Catechin, kaempferol, phloretin, phloridzin, quercetin and rutin (quercetin-3-rhamnosylglucoside) were obtained from Sigma Chemical Co. (PO Box 14508, St. Louis, MO, USA 63178-9916).

Quercetin-3-arabinose was obtained from Extrasynthese (Z.I. Lyon Nord, B.P. 62, 69730 Genay, France).

(-)-Epicatechin, epicatechin polymer, (+)-gallocatechin, procyanidin B2 and procyanidin B5 were kindly donated by Dr. L. Porter (Industrial Research Limited, Gracefield Research Centre, Lower Hutt, N.Z.).

Plant material

The apple cultivar used for most of the identification work was 'Lawfam', which was obtained from Havelock North Research Orchard, New Zealand. This was selected because an initial screening by TLC showed a large number of flavonoids and high concentrations of both anthocyanins and flavonols.

Samples of other apple genotypes and species, used for analysis of pigment composition, were obtained from a number of sources as detailed in Appendix A. Usually these samples were mature fruit collected at the time of normal harvest with collections being made in two seasons, 1989/90 and 1990/91.

Other apple tissues (bark, flesh, flower, leaf and seed) were obtained from 'Splendour' apple trees at Lincoln University Orchard.

TLC spray reagents

Aniline hydrogen phthalate - aniline (0.92 ml) and phthalic acid (1.6 g) were dissolved in *n*-BuOH (49 ml), ether (49 ml) and H_2O (2 ml).

Diphenyl-boric acid-ethanolamine complex (Naturstoffreagenz A) - 1% (w/v) solution in methanol.

Sodium carbonate - 1% (w/v) aqueous solution.

Vanillin-HCl - 5% (w/v) vanillin in ethanol was mixed with concentrated HCl in the ratio 4:1 just prior to use.

Methods

Handling of fruit

Skin was removed carefully from the fruit taking care to scrape off all underlying flesh with a scalpel. Samples that were not to be analysed immediately were weighed, snap frozen in liquid nitrogen, and stored at -80°C.

Extraction of anthocyanins and flavonols

Prepared apple skin was ground to a fine powder in a mortar and pestle using liquid nitrogen and extracted with 15.0% (v/v) acetic acid in methanol. The residue was re-extracted at least twice, with the same solvent, until all colour was removed and the combined extracts centrifuged at 5,000 g for 10 min. The supernatant was rotary evaporated at 40°C to near dryness and taken up in 0.5 to 1.0 ml of the extraction solvent. These crude extracts were centrifuged at 10,000 g for 5 min and the supernatant stored at -20°C until analysis by TLC or HPLC.

Extraction of proanthocyanidins

The above method of extraction, for the anthocyanins and flavonols, was followed except 100% methanol was used as the extracting solvent.

Separation of anthocyanins and flavonols

Extracts of anthocyanins and flavonols, prepared as above, were cleaned up and separated by polyamide column chromatography using a method adapted from Cheynier and Riguard (1986). A polypropylene column (1.0 x 12 cm) was packed by gravity flow with polyamide MN CC6 (Macherey-Nagel) and washed with eluants prior to addition of the sample. The sample (50-200 µl, depending on flavonoid concentration) was layered carefully onto the top of the column without disturbing the surface. The anthocyanins were eluted first with 15 ml of water/methanol/acetic acid (70:20:10, v/v/v). The flavonoids were then eluted with a linear gradient from 40 to 100% methanol in water (total volume of 50 ml), which was pooled as one fraction. Fractions were concentrated by rotary evaporation at 40°C to near dryness and then taken up in 200 µl of 15% (v/v) acetic acid in methanol. The samples were centrifuged at 10,000 g for 5 min and the supernatant stored at -20°C until analysis.

Thin layer chromatography

Initial separation of the flavonoids was carried out by two-dimensional TLC on plastic-backed Merck cellulose plates (No. 5577) using the following solvent systems:

(1) BAW - *n*-butanol:acetic acid:water (4:1:5)

(2) 15% HOAc - acetic acid in water

The TLC plates were then sprayed with one of the following solutions to visualize spots and characterize groups of compounds:

(**a**) Naturstoffreagenz A: The 3′,4′-dihydroxyflavonols appear orange and 4′-hydroxy equivalents produce yellow-green spots (Markham, 1982).

(**b**) Vanillin-HCl: The dry plates were sprayed and heated with a hairdryer. Red spots are produced immediately by catechins and proanthocyanidins and by flavanones and dihydroflavonols more slowly (Markham, 1982).

(**c**) Sodium Carbonate: Phloretin appears violet under UV and flavonols yellow, orange or brown (Gage *et al.*, 1951).

The R_f values of the individual compounds were calculated and compared to standards and published data (Harborne, 1959 & 1967; Mabry *et al.*, 1970) to make tentative identifications.

HPLC of flavonols and anthocyanins

A Waters 600 solvent delivery/control system with a Waters WISP 712 automatic sample injector and a Waters 490 variable wavelength ultraviolet detector were used to separate, identify and quantify the flavonoids. The column used was a 200 x 4.6 mm Brownlee Aquapore RP-18 fitted with a 15 x 3.2 mm guard column. Chromatographic traces were recorded using the Waters/Dynamic Solutions program 'Maxima'. Solvents used for elution were: (A) water containing 10.0% (v/v) acetic acid in water and (B) acetonitrile. Deaeration was achieved by vacuum filtration through a 0.22 μm filter, rapid sparging with helium (100 ml min^{-1} for 10 min) and constant slow bubbling of helium into capped, vented solvent reservoirs (5 ml min^{-1}). Samples (1-5 μl) were injected onto the column, which was maintained at 25°C using a Waters column heater. A flow rate of 1 ml min^{-1} was used with a linear 15 min solvent gradient from 5 to 20% B and a 15 min hold at the final concentration. The column was washed with 50% acetonitrile for 5 min then returned to the initial solvent composition and re-equilibrated for 10 min before the next analysis. The eluted components were detected at 350 nm for flavonols and 530 nm for anthocyanins. Individual compounds were identified and quantified by comparison of retention times with weighed amounts of known standards. Where standards were not available, quantification was based on an average value for that class of compound (e.g. quercetin glycosides), since responses were similar.

A preparative C_{18} column was used to isolate individual flavonoids and anthocyanins using the same conditions as above. Fractions, each containing a single peak, were collected and concentrated by rotary evaporation. These samples were then identified further by acid hydrolysis of the glycosides (Rosemary Webby, Industrial Research Limited, N.Z.). Compounds were also analysed by mass spectroscopy (MS) and proton nuclear magnetic resonance (NMR) and UV spectra data obtained (Dr. G. Lane, AgResearch, Palmerston North, N.Z.).

HPLC of proanthocyanidins

The system and column used was the same as for the flavonols and anthocyanins. Solvents used were: (A) water containing 10% (v/v) acetic acid, and (B) water. Samples (5-20 μl) were injected onto the column which was maintained at 70°C. A flow rate of 1 ml min^{-1} was used with a linear gradient

from 10-82% A in 47 min, 82-100% A in 8 min and then a hold at the final concentration for 5 min. The eluted compounds were detected at 280 nm for proanthocyanidins and 313 nm for phenolic acids. Individual compounds were identified and quantified as for the anthocyanins and flavonols.

Hydrolysis of flavonoid glycosides

To elucidate further the structures of the flavonoids, separated by preparative HPLC or TLC, they were hydrolysed to cleave the sugar from the aglycone. A flavonoid glycoside (up to 1 mg) was dissolved in 2-5 ml 2 M HCl:MeOH (1:1) in a boiling tube with a raindrop condenser on top. The solution was heated for 60 min on a steam bath and then evaporated to dryness on a rotary evaporator at 40°C. The residue was dissolved completely in a small volume of MeOH:H_2O (1:1) and the product was chromatographed (TLC - cellulose plates run in 15% HOAc) to determine whether hydrolysis had taken place.

To isolate the sugars and aglycone for further analysis the aqueous methanol solution was evaporated to half volume, then extracted several times with ethyl acetate (EtOAc) by vigorous shaking in a test-tube. The phases were separated with the aglycone in the EtOAc fraction while the sugars remained in the H_2O.

The aglycones were identified by cellulose TLC using the solvent BAW and spraying the dried plates with Naturstoffreagenz A, calculation of the R_f values and comparison with standards.

Analysis of the sugars was carried out by cellulose TLC with the solvent systems BAW or EPAW (EtOAc:Pyr:HOAc:H_2O, 36:36:7:21). The plates were sprayed with aniline hydrogen phthalate and heated in an oven at 100°C for about 5 min until brownish spots appeared. R_f values of the sample sugars were compared to known standards.

Comparison of flavonoid composition in different apple genotypes and species

Samples were prepared as outlined above, with three representative samples being taken as typical of each genotype or species. Samples were also representative of each apple containing both sun and shade sides of the fruit. For samples for the 1989/90 season, flavonols and anthocyanins in the samples were separated by polyamide column chromatography and analyzed by HPLC as described above. Samples from the 1990/91 season were not separated by column chromatography as this was not required due to improvements to the HPLC system with the acquisition of a variable wavelength UV detector. The system followed was identical to the previous method although a different column was used - a 220 x 4.6 mm Applied Biosystems Aquapore RP-18 fitted with a 18 x 3.5 mm Applied Biosystems Aquapore RP-18 Guard column.

Comparison of the two methods of quantification used indicated losses of 5-10% during the column chromatography step depending on the sample concentration. These losses were compensated for when calculating the final concentrations of the flavonoids for the 1989/90 season.

In the 1990/91 season the proanthocyanidins were also identified and quantified by the methods

outlined above.

Analysis of flavonoids in other apple tissues

Bark, flesh, flower, leaf and seed samples were taken from 'Splendour' apple trees and analyzed for all classes of flavonoids by TLC and HPLC, as for the skin samples, by the methods given above.

Statistical analysis

Concentrations of individual anthocyanins, flavonols and proanthocyanidins were calculated for each genotype or species. The percentage composition of the individual compounds within each of these groups was also calculated. ANOVA (using the method for samples of unequal sizes) was used to establish differences between genotypes for both concentrations and percentage composition of individual flavonoids. LSD's are given at the 5% significance level.

2.3 Results

Identification of the flavonoids

(a) Anthocyanins

Initial investigation of the apple anthocyanins, extracted from 'Lawfam' skin and separated on TLC, showed one major anthocyanin and three minor compounds (Figure 2.4 & Table 2.5). From the colour of these spots and the R_f value of the aglycone, after acid hydrolysis, they were all cyanidin glycosides. Spot A#1, the largest of the spots, cochromatographed with an authentic cyanidin-3-galactoside standard. From cochromatography it appears that spot #2 may be cyanidin-3-glucoside but it was difficult to determine as the R_f values of the cyanidin glycosides were close together.

HPLC analysis of the same extract was in agreement with the TLC results. The anthocyanins eluted off the HPLC column between the proanthocyanidins and the flavonols, with some slight overlap with the latter. The retention times were between 20 and 25 min. One very large peak, detected at 530 nm (Figure 2.5 & Table 2.6), co-eluted with cyanidin-3-galactoside standard. Because the cyanidin-3-galactoside was present in such a high amount, compared to the minor components, other peaks were only detected in samples with a high anthocyanin concentration or when a higher sample volume was injected. One of these minor HPLC peaks co-eluted with cyanidin-3-glucose standard. None of the other minor peaks could be identified due to lack of standards.

Further analysis of these anthocyanins by R. Webby confirmed these results and gave more information. She detected only three bands when the samples were run in TBA (*t*-butanol:acetic acid: water, 3:1:1, v/v/v). The lowest and largest of these bands being the cyanidin-3-galactoside as identified above. The sugar linkage and lack of acylation were confirmed. The highest running band contained some contaminant, probably flavonoid, but on hydrolysis it clearly gave cyanidin. Sugar analysis gave glucose with a trace of galactose. UV spectra were too weak to confirm linkages. Thus it can be confirmed only that this is a cyanidin glycoside, possibly cyanidin-3-glucose. The middle band was also weak but hydrolysis revealed it contained two anthocyanidins: cyanidin and a methylated cyanidin. Separation of the glycosides was not possible due to the sample being too small. Sugar analysis gave glucose and a trace of galactose, and as with the highest running band the UV spectra data was inconclusive. There was not enough material to determine whether these minor components were acylated or not.

(b) Flavonols

From initial screening by two-dimensional TLC six flavonol spots where detected (Figure 2.6 & Table 2.8). After spraying with Naturstoffreagenz A five of the spots reacted to produce an orange colour which is characteristic of the 3′,4′-dihydroxyflavonoids, whilst a small spot gave a yellow-green reaction and was thus identified as a 4′-hydroxyflavonoid. From other chromatographic data, including colour and R_f values, these were further identified as quercetin glycosides and a kaempferol glycoside respectively

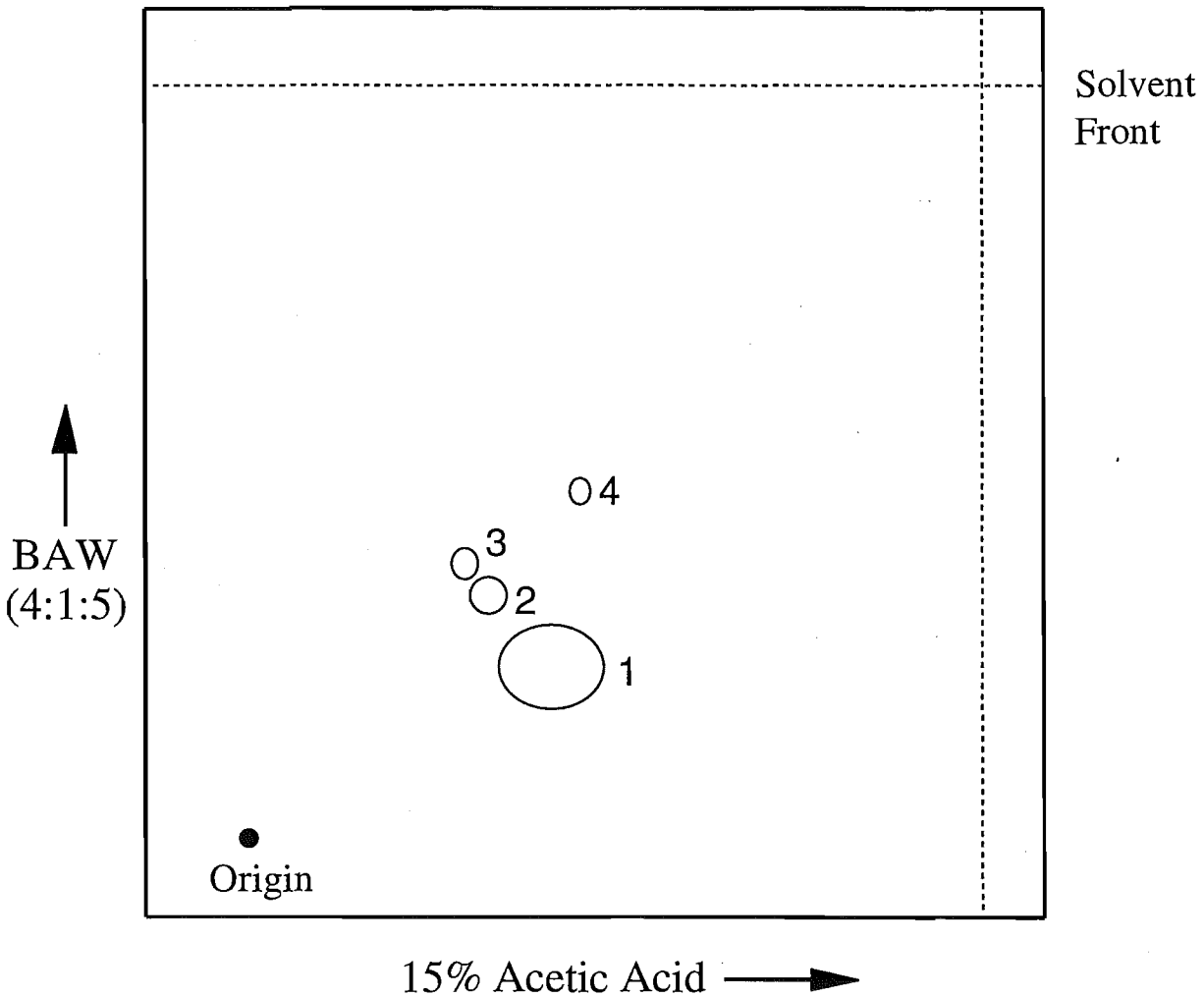


Figure 2.4: TLC of apple skin anthocyanins

Table 2.5: Chromatographic characteristics of apple skin anthocyanins (TLC)

Spot number	R_f BAW	R_f 15% HOAc	Colour	Colour in UV + NH_3	Probable identity
1	0.32	0.40	Pink	Blue	Cyanidin-3-galactoside
2	0.37	0.24	Pink	Blue	Cyanidin-3-glucoside?
3	0.39	0.22	Pink	Blue	Cyanidin glycoside
4	0.46	0.40	Pink	Blue	Cyanidin glycoside

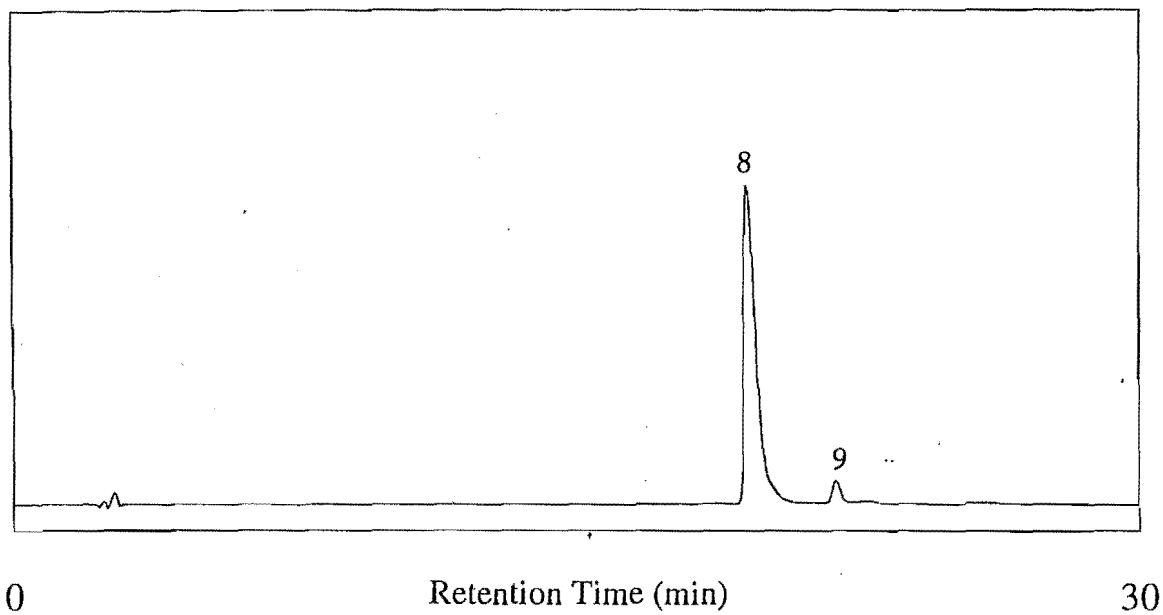


Figure 2.5: Typical HPLC trace for the anthocyanins extracted from a standard sample of apple skin (λ_{max} 530 nm).

Table 2.6: Retention times of the known anthocyanins of apple fruit on C_{18} RP-HPLC monitored at 530 nm (these retention times refer to the method give in Section 5.2.1)

Compound	Elution order	Anthocyanin	R. time (min)
A#1	8	Cyanidin-3-galactoside*†	19.9
A#2	9	Cyanidin-3-glucoside*	22.01

* Retention time matched authentic standard on HPLC
† Identified by hydrolysis and sugar analysis with authentic standards

(Table 2.8). Although identification of the specific quercetin glycosides is only tentative since many of the R_f values are similar and in some cases, such as the galactoside and glucoside, the spots overlap.

Subsequent HPLC analysis of the same flavonol fraction gave eight peaks which were labelled A-H for future reference (Figure 2.7 & Table 2.9). These were the last group of compounds to elute off the HPLC column and the retention times for these seven peaks were over a very narrow band from 22 to 26 min. When samples with a high concentration of flavonols were injected several smaller peaks were sometimes detected, with a longer retention time. However, with preparative scale HPLC only seven peaks were collected, since due to the higher loading one of the minor peaks (G) was not resolved under these conditions. With the preparative scale HPLC sample H also included the minor peaks with the longer retention time under standard HPLC conditions. Purity of the compounds varied greatly (Table 2.7) and in some cases (compounds C, E & H) these samples had to be separated further using TLC in order to make a valid identification. The identification of the compounds A to H was made as follows:

(A) Quercetin 3-O-galactoside - The retention time on HPLC matched an authentic standard. Hydrolysis of the preparative HPLC sample and analysis of the sugar confirmed this result with the position of the sugar linkage also confirmed by NMR (R. Webby).

(B) Quercetin 3-O-rhamnoglucoside - The retention time on HPLC matched an authentic standard. The sugar linkages were not confirmed by NMR but the sample cochromatographed with rutin, quercetin 3-O-rhamnosyl (1→6) glucoside.

(C) Quercetin 3-O-glucoside - The retention time on HPLC matched the standard. Sugar analysis of the hydrolysed sample also confirmed this to be a glucoside (R. Webby).

(D) Quercetin 3-O-xyloside - The retention time on HPLC matched the standard. Hydrolysis of the preparative HPLC sample and analysis of the sugar also confirmed this result (R. Webby).

Table 2.7: Purity of preparative HPLC fractions as estimated from relative A_{350nm} peak height using analytical HPLC

Fraction	R_f (min)	% Purity
A	17.2	95
B	18.2	Mixture
C	18.7	62
D	20.3	96
E	22.1	54
F	23.5	93
H	25.3	84

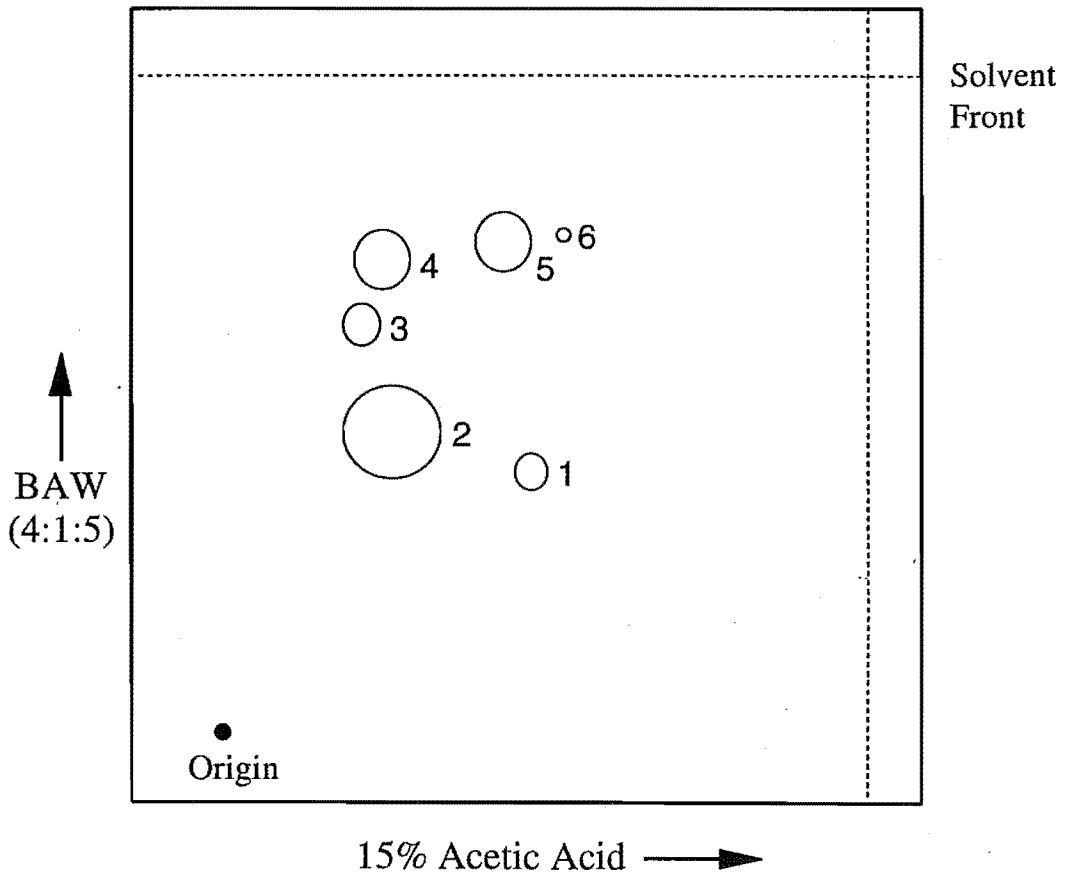


Figure 2.6: TLC of apple skin flavonols

Table 2.8: Chromatographic characteristics of apple skin flavonols (TLC)

Spot number	R_f BAW	R_f 15% HOAc	Colour in UV	Colour with spray (a)*	Probable identity
1	0.46	0.51	Brown	Orange	Quercetin-3-rutinoside
2	0.55	0.36	Brown	Orange	Quercetin-3-galactoside/ glucoside
3	0.65	0.31	Brown	Orange	Quercetin-3-xyloside
4	0.71	0.32	Brown	Orange	Quercetin-3-arabinoside
5	0.74	0.48	Brown	Orange	Quercetin-3-rhamnoside
6	0.76	0.54	Yellow	Green	Kaempferol glycoside

* Spray (a): Naturstoffreagenz A

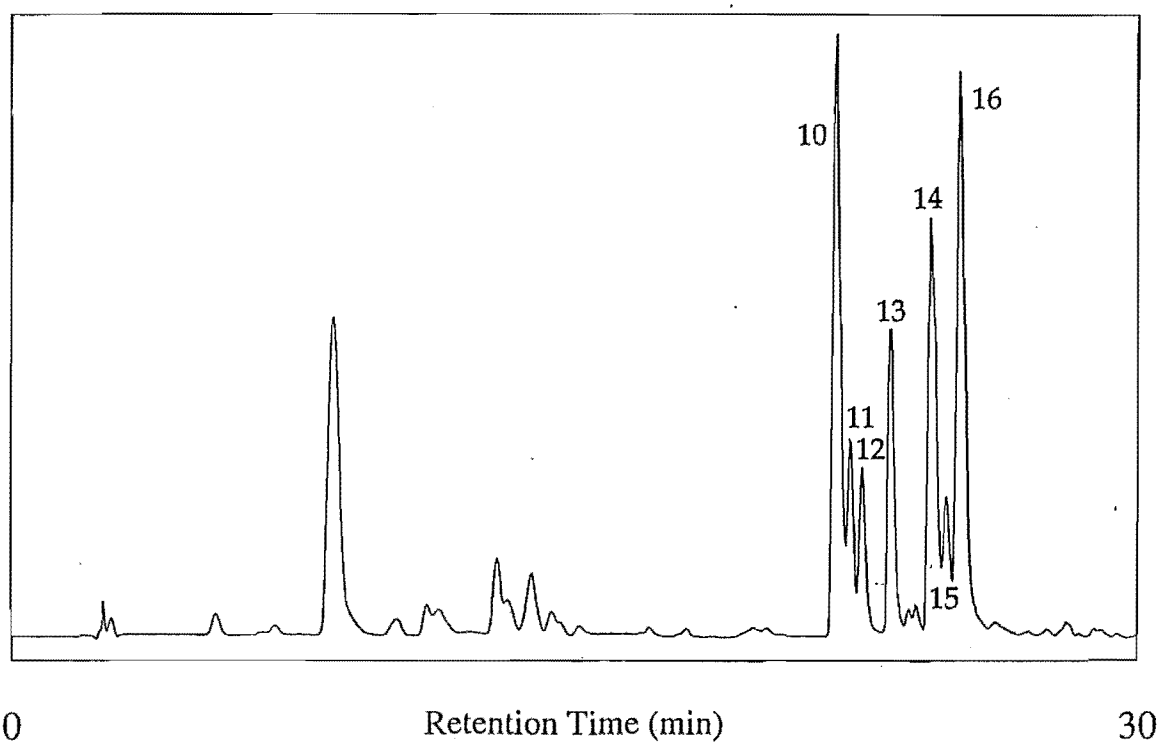


Figure 2.7: Typical HPLC trace for the flavonols extracted from a standard sample of apple skin (λ_{max} 350 nm)

Table 2.9: Retention times of the known quercetin glycosides of apple fruit on C_{18} RP-HPLC (these retention times refer to the method given in Section 5.2.1)

Compound	Elution order	Flavonol	R. time (min)
F#1 (A)	10	Quercetin-3-galactoside*†	22.2
F#2 (B)	11	Quercetin-3-rhamnoglucoside*†	22.5
F#3 (C)	12	Quercetin-3-glucoside*†	22.8
F#4 (D)	13	Quercetin-3-xyloside*†	23.6
F#5 (E)	14	Quercetin-3-arabinopyranoside‡	24.3
F#6 (F)	15	Quercetin-3-arabinofuranoside*†	24.6
F#7 (H)	16	Quercetin-3-rhamnoside*†	25.4

* Retention time matched authentic standard on HPLC & cochromatographed on TLC
† Identified by hydrolysis and sugar analysis with authentic standards
‡ Cochromatographed with authentic standard on TLC

(E) Quercetin 3-O-arabinopyranoside - There was not sufficient sample for sugar analysis but it cochromatographed with quercetin 3-O-arabinopyranoside.

(F) Quercetin 3-O-arabinofuranoside - The retention time on HPLC matched the standard. Arabinose was confirmed by sugar analysis of the hydrolysed sample and it also cochromatographed with the arabinofuranoside on TLC.

(G) Quercetin glycoside - This was a very minor peak and was not resolved by the preparative scale HPLC. A sample was obtained by PC but was too weak for sugar analysis and did not cochromatograph with any of the standards in the collection. It was not possible to identify this compound other than it was a quercetin glycoside. It may have possibly been an acylated glycoside since it ran in a different position to other standard glycosides.

(H) Quercetin 3-O-rhamnoside - The retention time for this matched the standard and cochromatographed with quercetin 3-O-rhamnoside; sugar analysis also gave rhamnose (R. Webby). Small amounts of the aglycone kaempferol were present in this sample (R. Webby), as was also noted previously from TLC results. Hydrolysis of this sample and analysis of the sugars indicated kaempferol 3-glucoside (astragalin) was definitely present and possibly the -3-rhamnoside (afzelin) and -3-rhamnoglucoside. Further attempts to identify kaempferol glycosides were made with extracts from *Malus sylvestris* which showed different minor HPLC peaks in the later regions although the quercetin glycosides were identical to the other cultivars. TLC showed three compounds the highest running spot in 15% HOAc appeared to be a kaempferol-3,7-glycoside. Analysis of the sugars gave rhamnose and galactose and a partial hydrolysis gave a glycoside which may be kaempferol-3-rhamnogalactose and a kaempferol-7-glycoside (probably rhamnoside). This suggests a possible structure of kaempferol-3-rhamnogalactoside-7-rhamnoside. One of the other compounds may be kaempferol-3-rhamnogalactoside but the other compound was too weak for analysis.

The fractions separated by preparative HPLC were also investigated by MS and NMR to provide structural evidence to complement the evidence from co-chromatography and hydrolysis. UV spectra data were also obtained for the individual compounds (A→H). This data is consistent with quercetin-3-glycosides being the dominant UV absorbing components eluted (Table 2.10). The long wavelength band of sample H is at a significantly shorter wavelength than for the other fractions, consistent with quercetin-3-rhamnoside being the main component. MS data showed that quercetin was the only aglycone present although there was some evidence for traces of phloretin glycoside, but these were not conclusive. Other aglycones such as naringenin were definitely absent. Taking the MS, NMR and UV spectra data together the following conclusions were made: compound (A) was a quercetin-3-ß-hexoside, (C) was a quercetin-3-hexoside (+ -pentoside), (D) quercetin-3-glycoside, (F) quercetin-3-pentoside and (H) quercetin-3-rhamnoside (Dr. G. Lane, pers. comm.). Insufficient quantities of the other samples were obtained to provide information. These results agree with the conclusions made from TLC, HPLC data and analysis of the products from acid hydrolysis.

Table 2.10: UV spectra (λ_{max} nm) of HPLC fractions and quercetin glycosides

Fraction/Compound	UV spectrum λ_{max} (nm)			
HPLC Fractions				
A	259	270sh	302sh	364
B	259	269sh	301sh	363
C	258.5	229sh	302sh	361
D	259	269sh	290sh	361
E	275sh			
F	258.5	268sh	294sh	359
H	258	266sh	287sh	351
Compounds				
Quercetin-3-galactoside[a]	257	269sh	299sh	362
-3-glucoside[b]	258			364
-3-rhamnoside[a]	256	265sh	301sh	350
-3-rutinoside[a]	259	266sh	299sh	359

[a] Data from Mabry *et al.* (1970)
[b] Data from Markham & Mabry (1975)

(c) Proanthocyanidins

Four distinct proanthocyanidin spots were detected by TLC after spraying with vanillin-HCl (Figure 2.8 & Table 2.11). One of the larger spots (#3) cochromatographed with (-)-epicatechin and another (#1) with procyanidin B2. The two smaller spots (#2 & #4) cochromatographed with procyanidin B5 and (+)-catechin respectively. A smear of vanillin reactive material (almost immobile in BAW) also showed on the TLC plates that did not correspond with any of the standards.

The proanthocyanidins were the first group of compounds eluted from the HPLC column with retention times from 5 to 20 min. A much larger number of compounds were detected at 280 nm by HPLC (Figure 2.9 & Table 2.12) than showed up on the TLC plates. However, some of these compounds could be discounted as proanthocyanidins and were probably phenolic acids, such as chlorogenic acid, which is present at high concentrations in apples. For the detection of phenolic acids the response at 313 nm is greater than at 280 nm, whereas for the proanthocyanidins the response is greater at 280 nm than 313 nm. By comparison of the two spectra some compounds could be eliminated. The two major peaks, P#2 and P#3, were identified by cochromatography (TLC) and the retention times on HPLC matched the

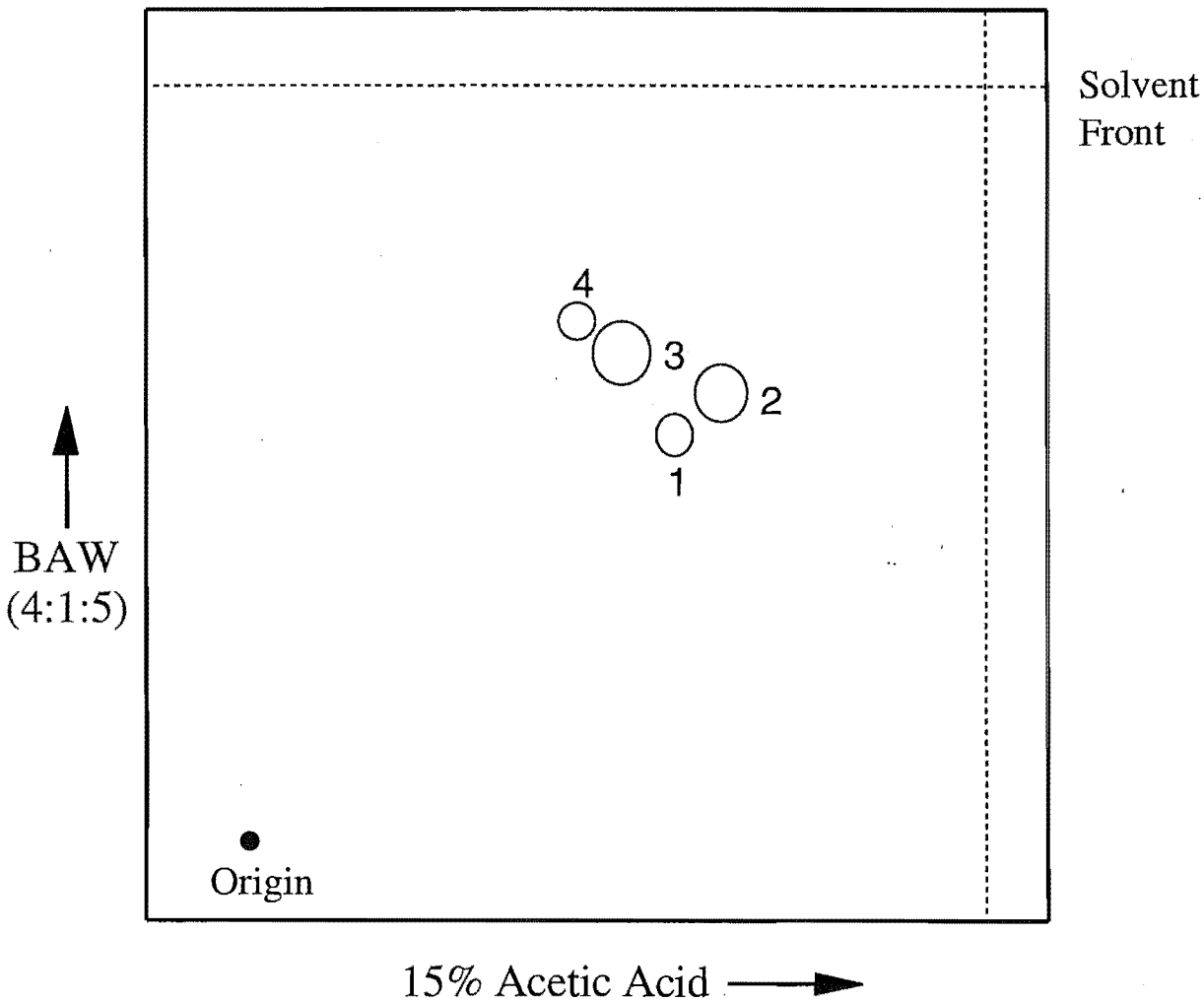


Figure 2.8: TLC of apple skin proanthocyanidins

Table 2.11: Chromatographic characteristics of apple skin proanthocyanidins (TLC)

Spot number	R_f BAW	R_f 15% HOAc	Colour with spray (ii)	Probable identity
1	0.55	0.61	Red	Procyanidin B5
2	0.61	0.68	Red	Procyanidin B2
3	0.67	0.55	Red	(-)-Epicatechin
4	0.71	0.50	Red	(+)-Catechin

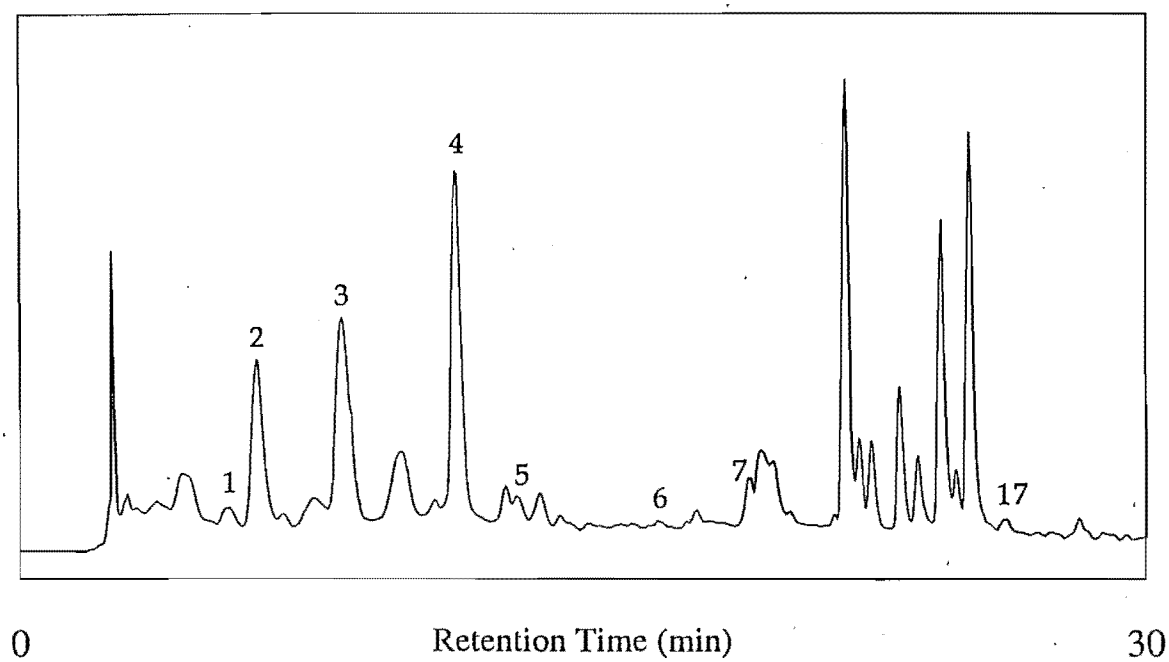


Figure 2.9: Typical HPLC traces for the proanthocyanidins extracted from a standard sample of apple skin (λ_{max} 280 nm).

Table 2.12: Retention times of the known proanthocyanidins of apple fruit on C_{18} RP-HPLC (these retention times refer to the method given in Section 5.2.1)

Compound	Elution order	Proanthocyanidin	R. time (min)
P#1	1	(+)-Catechin*	5.9
P#2	2	Procyanidin B2*	6.6
	3	Chlorogenic acid†	8.8
P#3	4	(-)-Epicatechin*	11.9
P#4	5	(+)-Gallocatechin*	13.9
	6	Dihydroquercetin†	17.6
P#5	7	Procyanidin B5*	19.6
	17	Phloridzin*	27.1

* Retention time matched authentic standard on HPLC & cochromatographed on TLC
† Retention time matched authentic standard on HPLC

authentic standards of procyanidin B2 and (-)-epicatechin. Three of the smaller peaks (P#1, P#4 and P#5) were also identified in the same way and were (+)-catechin, (+)-gallocatechin and procyanidin B5 respectively. Further attempts to identify the (+)-gallocatechin by ^{1}H-NMR and MS were inconclusive (G. Lane, pers. comm.). The main problem was the small quantity of (+)-gallocatechin able to be isolated from a sample of apple skin and impurities in the sample.

There are still some peaks from the HPLC chromatograms not yet identified, all of which are relatively minor and individually account for less than 5% of the total proanthocyanidins. These components appear to be proanthocyanidins since they gave positive results with the vanillin test. However, they were not able to be identified further due to the lack of standards and also the small quantities present in the apple skin.

(d) Other Flavonoids

The only other flavonoid detected in apple skin by TLC and HPLC was the dihydrochalcone phloridzin. Retention time of this compound matched the standard on the HPLC and it also cochromatographed on TLC. This was the last flavonoid compound to be eluted from the HPLC column with a retention time of 27 min and detection was made at 280 nm (Figure 2.9 & Table 2.12). After acid hydrolysis the aglycone gave a positive result for phloretin on TLC, when sprayed with sodium carbonate, and analysis of the sugar gave glucose. Phloridzin, both standard and sample, also gave a positive test when sprayed with vanillin.

Occasionally, very small traces of the dihydroflavonol dihydroquercetin were detected on the HPLC. This was detected at 280 nm and eluted in the middle of the proanthocyanidins, with a retention time of 17.6 min. The dihydroquercetin was identified only by the matched retention time with an authentic standard. It was not detected on the TLC plates but this was probably due to the low concentrations present.

Composition and quantification of the flavonoid groups in apple genotypes and species

Details of the flavonoids, and the concentrations, detected in 39 New Zealand-grown apple genotypes and species are given in Appendix B. As postulated only four classes of flavonoids were detected in apples, these being anthocyanins, flavonols, proanthocyanidins and a dihydrochalcone. Only the flavonols and proanthocyanidins were present in all the apples at maturity and the anthocyanins and dihydrochalcone were present in only some genotypes and species.

(a) Anthocyanins

There were no qualitative differences in the composition of the anthocyanins in the various apple genotypes and species examined but there were quantitative differences. The total anthocyanin levels at maturity varied from zero in few non-red genotypes, such as 'Granny Smith', through to a maximum of

4.39 mg g^{-1} FW in 'Oregon Red' in 1991 (Appendix B). There were also significant quantitative differences in the individual anthocyanins although when calculated in terms of percentage composition these differences were not as great. In all the genotypes and species examined cyanidin-3-galactoside was the predominant anthocyanin generally accounting for 90-95% of the total anthocyanins (see Appendix C). In some genotypes and species cyanidin-3-galactoside was the only anthocyanin detected but in all these samples the total concentration was less than 100 μg per gram fresh weight; if there were traces of the minor components they were below the detection level. The remaining anthocyanins (5-10%) consisted of one to three minor components, being various cyanidin glycosides as outlined earlier. In only one cultivar, 'Ikorokavka Alajah', was there any difference; an additional HPLC peak later eluting than the others was detected. However this still accounted for only 3% of the total anthocyanins. There were some significant differences in the percentage composition of the anthocyanins for the different genotypes and species. Those genotypes that were measured in both seasons showed no significant differences between the years.

(b) Flavonols

The same flavonol glycosides were present in the different apple genotypes and species, but at different levels. As with the anthocyanins there were wide variations between the various genotypes and species in both total flavonol content and concentrations of individual quercetin glycosides (Table 2.13 and Appendix B). Total flavonol levels ranged from a low of 0.62 mg g^{-1} FW in *Malus sylvestris* (a close relation of the commercially grown *Malus pumila*) through to a maximum of 10.15 mg g^{-1} FW in 'Lawfam' in 1991. The pattern of distribution of the flavonol glycosides was generally similar in the different genotypes and species. This could be seen when the individual flavonol concentrations were converted to percentage composition (Appendix C). The variation was not as great but there were still significant differences between some genotypes and species. However, there were no significant differences in percentage composition for the same genotypes measured in both seasons. In most genotypes and species quercetin 3-galactoside was the predominant flavonol comprising on average 39% of the total flavonol. This was followed by the arabinofuranoside (19%), and then a group including the rhamnoside (12%), glucoside (12%), and the xyloside (10%). The final group included the minor components which were the rutinoside, arabinopyranoside and an unknown quercetin glycoside each making up about 1.5% of the total. The sum of the other unidentified flavonols on average accounted for 3% of the total and this comprised a small number of kaempferol glycosides, whose identity was discussed earlier. This group showed the biggest variation in percentage composition, probably because individually the components were at a very low concentration and were generally only detected in genotypes or species that had a high total pigment concentration. These compounds may have been present at very low concentrations in the other genotypes but below detection levels. The fact that they were not detected is probably not important since they were present at too low a concentration to be of significance for colour production; this is discussed in Section Three. There were no major differences in the composition of

Table 2.13: Composition of the flavonol glycosides in apple skin determined by HPLC

Quercetin Glycoside	Concentration (mg g^{-1} FW)			Percentage composition		
	Min.	Max.	Average	Min.	Max.	Average
Galactoside	0.129	4.184	1.791	20.68	52.19	38.89
Rutinoside	0.005	0.511	0.058	0.49	3.50	1.43
Glucoside	0.038	1.700	0.618	4.39	24.34	12.34
Xyloside	0.076	1.276	0.459	5.69	14.84	10.06
Arabinopyranoside	0.007	0.173	0.072	0.74	2.54	1.57
Arabinofuranoside	0.203	1.795	0.809	11.40	32.50	18.64
Unknown	0.011	0.165	0.068	0.74	4.01	1.59
Rhamnoside	0.033	1.729	0.572	5.25	24.98	12.38
Other	0.00	0.865	0.147	0.00	17.29	3.09
Total	0.624	10.146	4.595	-	-	-

these minor compounds. Although the pattern of glycosylation was similar for most genotypes or species there were a few minor differences. In two of the apple types examined, *Malus sylvestris* and Maling 9 rootstock, quercetin-3-galactoside was not the predominant sugar. In these cases the arabinofuranoside was present in the highest concentration but it was followed by the galactoside.

(c) Proanthocyanidins

The proanthocyanidins also showed considerable variation in both total concentration and the concentrations of the individual compounds (Appendix B). Total proanthocyanidin levels, in those genotypes measured, ranged from 1.31 mg g^{-1} FW in 4 last up to 6.26 mg g^{-1} FW in 'Oregon Red'. Like the flavonols the percentage composition of each component was more consistent (Table 2.14 & Appendix C) although there were still some significant differences. The main proanthocyanidins in most apple skins were (-)-epicatechin and procyanidin B2 comprising on average 35% and 30% of the total respectively. Procyanidin B5 accounted for, on average, 4% of the total proanthocyanidins, while (+)-catechin and (+)-gallocatechin (putative) were 2.5% each. Other proanthocyanidins together comprised 26% of the total; the identity of these compounds was discussed earlier. This group comprised from four to twelve compounds each accounting for less than 5% of the total proanthocyanidin content. Four of the peaks were common between the genotypes but there were slight differences in the others. These are probably of little significance since they are such minor components and are probably closely related biosynthetically.

The pattern of proanthocyanidin composition was the same for most genotypes but there were some

Table 2.14: Composition of the proanthocyanidins in apple skins determined by HPLC

Proanthocyanidin	Concentration (mg g^{-1} FW)			Percentage composition		
	Min.	Max.	Average	Min.	Max.	Average
Catechin	0.001	0.115	0.057	0.02	7.38	2.57
Epicatechin	0.347	2.043	0.923	26.57	46.03	35.30
Gallocatechin	0.016	0.238	0.062	0.80	4.68	2.30
Procyanidin B2	0.390	1.684	0.744	21.84	38.36	29.72
Procyanidin B5	0.008	0.443	0.116	0.49	7.08	4.18
Other	0.174	1.848	0.682	11.20	31.59	25.93
Total	1.306	6.258	2.583	-	-	-

significant differences. In most cultivars (-)-epicatechin was the predominant proanthocyanidin but in five genotypes (4 last, 4/17, 92 Just, 'Granny Smith' and 'Ikorokavka Alajah') procyanidin B2 was present in higher concentrations. In 'Oregon Red' (+)-catechin was almost absent and in 'Lawfam' there was very little procyanidin B5.

(d) Other flavonoids

The dihydrochalcone phloridzin was the only other flavonoid regularly detected in the skin of the apple genotypes and species. The levels of phloridzin varied from undetectable in many genotypes and species to a maximum of 60.91 μg g^{-1} FW in J997 (Appendix B). In all the genotypes and species examined it was a minor component, accounting for less than 1 % of the total flavonoids. No other dihyrochalcones were detected in any of the apples, although it is possible that they were present in trace amounts as one of the unidentified components.

Distribution of flavonoids in different apple tissues

There was considerable variation in the levels of flavonoids present in the different apple tissues. The leaves, flowers and fruit skin had the highest concentrations with lower concentrations in the seeds and only small amounts in the bark and flesh (Table 2.15). The same compounds were present in the different tissues but the relative proportions of these to the total flavonoids varied between tissue types.

(a) Anthocyanins

Apart from the skin, anthocyanins were present only in the flowers in reasonable concentrations

Table 2.15: Composition of the flavonoids in apple tissue types determined by HPLC

Flavonoid	Concentration (mg g^{-1} FW)					
	Bark	**Flesh**	**Flower**	**Leaf**	**Seed**	**Skin**
Anthocyanin						
Cyanidin-3-galactoside	*t*	-	0.100	*t*	-	0.795
Other[a]	-	-	-	-	-	0.041
Dihydrochalcone						
Phloridzin	0.254	0.137	5.581	15.270	1.763	*t*
Other[a]	0.028	-	0.121	0.385	-	-
Flavonols						
Quercetin-3-galactoside	0.012	*t*	1.884	0.986	*t*	1.519
-3-rhamnoglucoside	-	-	*t*	*t*	-	0.025
-3-glucoside	*t*	-	1.402	0.458	*t*	0.526
-3-xyloside	*t*	-	0.287	0.385	*t*	0.398
-3-arabinopyranoside	-	-	*t*	*t*	-	0.054
-3-arabinofuranoside	0.011	-	0.783	0.735	*t*	0.787
unknown	-	-	-	-	-	0.088
-3-rhamnoside	0.022	*t*	1.114	0.851	*t*	0.910
Other[a]	*t*	-	0.247	0.179	-	0.105
Proanthocyanidins						
(+)-Catechin	*t*	*t*	-	-	-	0.047
Procyanidin B2	*t*	0.019	*t*	-	-	0.946
(-)-Epicatechin	*t*	0.091	*t*	-	-	1.134
(+)-Gallocatechin	*t*	-	-	-	-	0.052
Dihydroquercetin	*t*	-	*t*	-	-	*t*
Procyanidin B5	*t*	*t*	*t*	-	-	0.109
Other[a]	*t*	-	*t*	-	-	0.873
Total Flavonoid	0.210	0.114	11.521	19.250	1.765	8.411

- Not detected
t Trace (<0.01 mg g^{-1} FW)
[a] Concentration represents the total of all other compounds

whilst traces sometimes occurred in the leaves and bark. The predominant anthocyanin in all tissues was cyanidin-3-galactoside and only in the skin were any other cyanidin glycosides detected, although this may have been due to the low concentrations in the other tissues. No anthocyanins were present in the flesh of 'Splendour' apples and in most cultivars and species examined they were absent with the flesh being white, yellow or green (Appendix A). However in *Malus pumila niezwetzkyana* they were noted as present in the flesh giving it a pale pink colour.

(b) Flavonols

Flavonols were detected in all tissues at varying concentrations, from the flesh where they were present only in trace amounts to the leaves and flowers where levels reached up to 3.59 and 5.72 mg g^{-1} FW, respectively. Quercetin glycosides were dominant in all tissues and traces of various kaempferol glycosides were detected only in tissues were flavonols were present in high concentrations (flowers, leaves and skin). There were some variations in the pattern of glycosylation in the different tissues, for example the glucoside was present at a relatively higher concentration in the flower than in other tissues. Quercetin-3-galactose was the dominant glycoside in all tissues except the bark where quercetin-3-rhamnoside was dominant.

(c) Proanthocyanidins

Proanthocyanidins were present mostly in the fruit skin but were also detected in other tissues such as the bark, flesh and flowers, although none were detected in the leaves or seeds. The composition of the individual proanthocyanidins was similar throughout the different tissues, with (-)-epicatechin and procyanidin B2 being dominant apart from the flower where procyanidin B5 was high. Some of the unidentified proanthocyanidins observed in the skin also occurred in the other proanthocyanidin containing tissues.

(d) Other flavonoids

The dihydrochalcone phloridzin was ubiquitous in apple tissue and was the predominant individual flavonoid in all tissues but the fruit. Concentrations of phloridzin were very high in the flower (6.6 mg g^{-1} FW) and leaf (up to 12 mg g^{-1} FW) but lower in the bark (0.25 mg g^{-1} FW) and seed (1.8 mg g^{-1} FW). Smaller quantities were detected in the flesh but there were only traces in the skin. However, further analysis of the flesh showed that much higher concentrations of phloridzin were present in the outside of the flesh compared to the inside of the apple. Thus, if care is not taken in preparing skin samples for analysis, by removal of all underlying cortical tissue, 'contamination' may occur. This could result in elevated phloridzin levels and lower flavonol levels being estimated for the skin. Several other compounds were present in the flower, leaf and bark samples which had a similar retention time on the HPLC and similar UV spectra to phloridzin. These are probably other phloretin glycosides but they were not identified any further.

2.4 Discussion

Separation of the flavonoids

It was found that HPLC separation of the apple flavonoids was a major improvement over TLC separation with greater resolution of the individual quercetin glycosides. With TLC some glycosides cochromatographed so there appeared to be only five quercetin glycosides present but which on HPLC gave eight. Neverthehless TLC is still useful for quick screening of the types of flavonoid compounds present. It can also provide useful data for the identification of compounds, such as R_f values and by the colours of the compounds with the use of spray reagents like Naturstoffreagenz A. TLC was also used here to complement data obtained from HPLC in the identification of the flavonoids.

The HPLC system developed was an improvement over many previously reported in that all the quercetin glycosides were separated, including the galactoside and glucoside which have often been difficult to resolve (Dick *et al.*, 1987). No peak was found to contain more than one compound although as the column aged there was some loss of resolution of peaks; small peaks such as quercetin-3-rutinoside (F#2 in Figure 2.7) merged into the quercetin-3-galactoside peak (F#1). Improvements were made to the method throughout the period of study, particularly with the acquisition of the variable wavelength detector which allowed all compounds to be quantified in a single run. The retention times varied slightly with the differences in methods but the order of elution remained the same. The elution order was similar to those reported by Dick *et al.* (1987) and Oleszek *et al.* (1988).

This system is applicable to many other plant species where a different range of flavonoids may be present. Slight changes may have to be made to the gradient and run times to get an appropriate spread of compounds depending on the elution times of those present.

Identification of the flavonoids

(a) Anthocyanins

A summary of the anthocyanins identified in this study from the skin of New Zealand-grown apple genotypes and species compared with what has previously been observed is shown in Table 2.16. Identification of the predominant anthocyanin in apple skin agrees with the findings of previous researchers that it is cyanidin-3-galactoside (Duncan & Dustman, 1936; Sando, 1937; Walker, 1964; Sun & Francis, 1967). Because the minor compounds are present in such low levels in the plant it is difficult to be sure how many compounds were present. There were definitely three cyanidin glycosides present in apple skins: cyanidin 3-*O*-galactoside, a methylated cyanidin glycoside and another cyanidin glycoside (probably the 3-glucoside), but there may be at least another cyanidin glycoside depending upon how much overlap exists between bands obtained by TLC. No arabinosides were detected although they have previously been reported (Sun & Francis, 1967; Timberlake & Bridle, 1971). This would suggest either that the composition is influenced by external factors or, more likely, it was an artifact of paper

Table 2.16: Summary of the anthocyanins found in New Zealand-grown apples compared to those reported previously in apple skin

Anthocyanin	Noted previously	This study
Cyanidin-3-arabinoside	✓	-
-arabinosylgalactoside	✓	-
-7-arabinoside	✓	-
-3-galactoside	✓	✓
-3-galactoside (acylated)	✓	-
-3-gentiobioside	✓	-
-3-glucoside	✓	✓
-3-glucoside (acylated)	✓	-
-3-xyloside	✓	-
Methylated cyanidin glycoside	-	✓

✓ Present
- Absent

chromatography (Timberlake *et al.*, 1971). These minor anthocyanins represent approximately 5 to 10% of the total anthocyanins which is similar to the figures reported by Timberlake & Bridle (1971).

Cyanidin 3-galactoside is distinctive of the two Pomoideae, *Malus* and *Pyrus*, and is generally absent from the other sub-families of the Rosaceae (Harborne, 1967). No delphinidin or its relatives were present and they are completely absent from the Rosaceae. Pelargonidin was also absent and is rare in the Rosaceae, appearing only in mutants of rose and raspberry (Harborne, 1967). Since all the anthocyanins present in apples are cyanidin glycosides they are not significantly different in terms of the biosynthetic pathway and differ only in the final glycosylation stages.

(b) Flavonols

The composition of the flavonols in the skin of New Zealand-grown apple genotypes and species is very similar to that which has been reported in overseas studies, with only minor differences (Table 2.17). As expected, quercetin glycosides were the predominant flavonols and most of those detected have been reported previously. However, there are eight glycosides reported here in comparison to the five or six reported previously together in one cultivar (Siegelman, 1955; Oleszek *et al.*, 1988; Burda *et al.*, 1990). The quercetin-3-arabinopyranoside (guaijaverin) has not been reported previously from apples but has been found before in other plants (Harborne & Williams, 1975). Dick *et al.* (1987) reported only the

Table 2.17: Summary of the flavonols found in New Zealand-grown apples compared to those reported previously in apple skin

Flavonol	Noted previously	This study
Quercetin-3-arabinofuranoside	✓	✓
-arabinopyranoside	-	✓
-3-diglucoside	✓	?[a]
-3-galactoside	✓	✓
-3-rhamnoside	✓	✓
-3-rutinoside	✓	✓
-3-xyloside	✓	✓
Kaempferol glycosides	✓[b]	✓

✓ Present
- Absent
[a] Possibility only (see discussion)
[b] Reported by only two groups previously

3-arabinofuranoside but found no evidence for the occurrence of other isomers of quercetin arabinoside in their apple extracts. However, there were unidentified minor peaks in their HPLC traces and since it is only present in low concentrations it may have been overlooked in this and previous studies. The other quercetin glycoside detected here was not identified further since it was present in such low concentrations. This could possibly be the quercetin diglucoside (meratin) that was reported by Fisher (1966) but little chromatographic data is available to confirm this. As with most other reports free quercetin was not detected and this indicates a high level of glycosylation activity.

Kaempferol glycosides were definitely present but at very low levels and with a number of different glycosides possibly present. It is interesting to note that these appear to have a different glycoside composition to the quercetin glycosides with rhamnose and glucose being the main sugars rather than galactose. It is also possible that there was glycosylation at the '7'-position, in addition to the '3' position, which was not observed in the quercetin glycosides. Studies of the sugar specificity of the glycosyltransferases may help to determine which kaempferol glycosides are likely to be present. These kaempferol glycosides were the only compounds, apart from phloridzin, with only the 4′-hydroxylation pattern suggesting that the activity of the 3′-hydroxylase was high. No myricetin was found and flavonols with the 3′,4′,5′ hydroxylation pattern are uncommon in the Rosaceae (Harborne, 1967).

(c) Proanthocyanidins

In general the apple skin proanthocyanidins that were identified here agreed with those reported previously (Table 2.18). Again these show the predominance of the 3′,4′-hydroxylation pattern with (-)-epicatechin and its derivative, procyanidin B2, present in high concentrations. Procyanidin B5 has not been reported previously in apple skin but has been found in cider (Lea & Timberlake, 1974) so its presence was not unexpected. Its presence is also logical since it is another (-)-epicatechin dimer and this was the major compound present.

The only anomaly was (+)-gallocatechin which was detected, although not conclusively identified. This has been reported previously, by a number of workers, as a minor component in apples (Mosel & Herrmann, 1974a & b; Prabha & Patwardhen, 1985a). Although (-)-epigallocatechin has been reported previously it was not identified here since no standard was available for comparison of retention time on HPLC. This could possibly account for another of the unidentified compounds likely to be present in only trace amounts. The presence of these two compounds in apples is unusual since, as mentioned earlier, the 3′,4′,5′ hydroxylation pattern is uncommon in the Rosaceae. The anthocyanins and flavonols with the equivalent tri-hydroxylation pattern (e.g. delphinidin and myricetin) have not been detected.

Several other proanthocyanidins were detected but could not be identified. It is likely that these were other procyanidins (dimers, trimers and larger) based predominantly on (-)-epicatechin but may also contain (+)-catechin and possibly the flavan-3,4-diol. Included in these are probably procyanidin B1 and C1 since they have been commonly reported together with procyanidin B2 and (-)-epicatechin in other plants (Haslam, 1982). These three compounds were found in cider by Lea & Timberlake (1974).

Table 2.18: Summary of the proanthocyanidins found in New Zealand-grown apples compared to those reported previously in apple skin

Proanthocyanidin	Noted previously	This study
(+)-Catechin	✓	✓
(-)-Epicatechin	✓	✓
(-)-Epigallocatechin	✓[a]	-
(+)-Gallocatechin	✓	✓
Procyanidin B2	✓	✓
Procyanidin B5	-	✓

✓ Present
- Absent
[a] Noted in only two cultivars

(d) Other flavonoids

All species of the genus *Malus* contain dihydrochalcones with phloridzin being the most common (Macheix *et al.*, 1990). It has been stated that phloridzin was located in the leaves, stems and seeds but absent from the apple fruit. Later research by a number of groups of workers has disproved this (Dick *et al.*, 1987; Oleszek *et al.*, 1988) and it was also detected in our samples (Table 2.19). However phloridzin was present in trace amounts in New Zealand apple skin samples in contrast to the report by Burda *et al.* (1990) where the dihydrochalcones made up to 15% of the total flavonoids. Dick (1986) also reported levels up to 330 $\mu g\ g^{-1}$ FW in fruit skins of Canadian apple cultivars. Other workers have reported much lower levels (Coseteng & Lee, 1987).

Apart from phloridzin no other phloretin glycosides were detected in the fruit skin samples, although phloretin xylogalactoside and phloretin xyloglucoside have been reported previously (Oleszek *et al.*, 1988; Burda *et al.*, 1990). This difference could be due to sample preparation as discussed earlier. Other explanations for these differences could be due to environmental factors or the developmental stage of the fruit. Most of the present identification work was based on mature fruit and it is possible that phloridzin and other glycosides were present in higher proportions in immature fruit. This possibility is examined in Section Four where developmental changes were studied.

Relationships between flavonoid classes

The biosynthetic relationship between the flavonoid classes has been discussed by a number of researchers (Harborne, 1988; Heller & Forkmann, 1988; Stafford, 1990). Based on these and on the evidence from the compounds present a biosynthetic scheme for the flavonoids in apples has been proposed (Figure 2.10); this is discussed in more detail in Section Five. The pathway does not include the synthesis of (+)-gallocatechin which is analogous to (+)-catechin but has the 3′,4′,5′ hydroxylation

Table 2.19: Summary of the dihydrochalcones found in New Zealand-grown apples compared to those reported previously in apple skin

Dihydrochalcone	Noted previously	This study
Phloretin xylogalactoside	✓	-
Phloretin xyloglucoside	✓	-
Phloridzin	✓	✓

✓ Present
- Absent

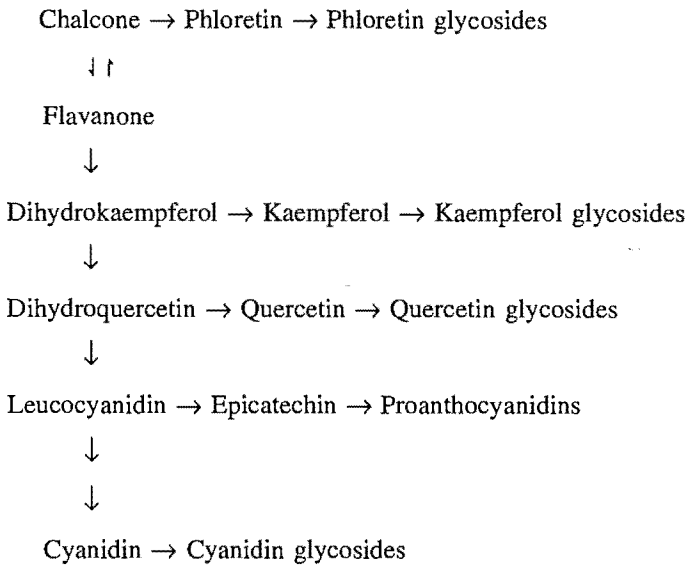


Figure 2.10: Proposed biosynthetic relationship of the flavonoid groups in apples

pattern on the B-ring. There are no other compounds with the equivalent hydroxylation pattern, such as the flavonol myricetin or the anthocyanin delphinidin, seen in apples. All other compounds have only the 3′,4′ hydroxylation pattern with the exception of the traces of kaempferol and phloretin glycosides which have only the 3′ hydroxylation of the B-ring. Apart from the occasional detection of very small amounts of dihydroquercetin none of the other possible intermediates in this pathway, such as chalcone or flavanone glycosides (e.g. eriodictyol), were detected. The presence of these may have given clues as to the stage at which 3′-hydroxylation occurs. All that can be deduced is that hydroxylation probably occurs after the chalcone, since phloretin was not hydroxylated in the 3′ position. This step is also probably after dihydrokaempferol is produced, since some kaempferol glycosides were detected. The probable step is the conversion of dihydrokaempferol to dihydroquercetin since all other compounds after this point, except (+)-gallocatechin, show the 3′,4′-hydroxylation pattern. This is a common conversion by the enzyme flavonoid 3′-hydroxylase which can also convert kaempferol to quercetin besides conversion of the earlier compounds (Heller & Forkmann, 1988). Thus further enzyme studies are required to elucidate the actual point of hydroxylation.

Composition and quantification of the flavonoid groups in apple genotypes and species

As was stated earlier four classes of flavonoids were detected in 39 apple genotypes and species examined, these were anthocyanins, flavonols, proanthocyanidins and a dihydrochalcone, although only the flavonols and proanthocyanidins were present in all apples sampled. However it is possible that the other compounds may be present at different developmental stages since these measurements were taken

only at maturity. For example it has been noted that many non-red apple cultivars do contain anthocyanins in the immature fruit but these disappear as the fruit develops (Lancaster, 1992). Changes in flavonoid composition during development are examined in Section Four. Unlike some plants it was not possible to distinguish different genotypes, or even those species examined, on the basis of flavonoid composition.

The percentage composition of the anthocyanin glycosides was consistent in all genotypes and species, with cyanidin-3-galactoside comprising 90-95% of the total and other cyanidin glycosides the remaining 5 to 10%. This is similar to the figures of 83-94% reported by Timberlake & Bridle (1971) for the galactoside. However it is different from some others such as Sun & Francis (1967) who reported that the galactoside comprised only 68% of the total anthocyanins. Mazza & Velioglu (1992) also reported that the galactoside only accounted for 39% of the total with the other glycosides present in higher concentrations in the one apple species. Although the same species were examined here the galactoside still accounted for 93%. The reasons for these differences are not clear, but some variation may be due to differences in quantification methods. It may also be possible that glycoside patterns are influenced by environmental factors but to what extent has not been determined.

The composition of the quercetin glycosides was also consistent in the different apple genotypes and species. In all but two apple types quercetin-3-galactoside was predominant as has been reported previously for a range of cultivars (Sando, 1937; Workman, 1963; Walker, 1964). However quercetin-3-rhamnoside was reported as the predominant glycoside in some cultivars including 'Golden Delicious' (McRae *et al.*, 1990). For the 'Golden Delicious' fruit sampled in this study the rhamnoside only comprised 18% of the total with both the galactoside and arabinopyranoside present in higher amounts. As with the anthocyanins the reasons for these differences are not clear.

The main variation between the genotypes and species was in the total levels of each group (anthocyanins, flavonols and proanthocyanidins) and in the concentrations of the individual compounds. Concentrations of flavonoids detected in the skin of the apple genotypes studied were higher than most of those previously reported. Quercetin levels were between 0.6 and 10 mg g^{-1} FW whereas previously reported values generally fell between 1 and 2 mg g^{-1} FW (Workman, 1963; Burda *et al.*, 1990; McRae *et al.*, 1990). The only exception was studies of New Zealand-grown cultivars by Walker (1964) who reported levels that were analogous, although some were still higher, to those reported here at between 0.4 and 18.8 mg g^{-1} FW. The proanthocyanidin values obtained were between 1.3 and 6.3 mg g^{-1} FW and other reported values have been 1.1 to 1.4 mg g^{-1} FW (Mosel & Herrmann, 1974a & b; Burda *et al.*, 1990; McRae *et al.*, 1990). The phloridzin levels reported here were between 0 and 61 μg g^{-1} FW, which are analogous to those reported by Coseteng & Lee (1987) but significantly lower than those given by Burda *et al.* (1990) and Dick (1986). However only levels at maturity were reported here and the levels may be higher in the immature fruit. This is highly probable since it would account for the bitterness of the immature fruit of many genotypes and could also possibly prevent the fruit from being eaten before the seeds are fully developed and ready for dispersal.

Since most of these values are from different genotypes, grown at different locations with different environmental factors and taken at different maturity stages, it is difficult to specify the causal factors. It is well known that many different factors are concerned in the development of colour in apples and include light, humidity, temperature and cultural factors and treatments (Walter, 1967). Since Walker (1964) also found the same high flavonoid levels in his study of New Zealand apples it would suggest some factor particular to the local environment or cultural practices. Light may be a key factor since New Zealand has very high incident light levels with a high UV component which is known to increase flavonoid concentrations (Walter, 1967). The levels observed by Walker (1964) for many cultivars were higher than those reported here which may be due to the experimental method employed. The simple spectrophotometric estimation of flavonoid concentration used by Walker is prone to interference from other compounds (Herrmann, 1976). With the HPLC method used here accurate determinations of each of the quercetin glycosides could be made.

In most cases there were no significant differences in the flavonol and anthocyanin levels of genotypes which were measured in both seasons. The differences that did occur were possibly due to different environmental conditions, as mentioned above, or differences in maturity at sampling (as was the case with two Fuji samples). Those genotypes with similar parentage, or that were sports of the same genotype, also showed similarities. 'Gala' related genotypes ('Regal Gala', GS 150, 4/1, 5/22, 5/30, 3/16 & 4/7) showed no significant differences in flavonol levels but there were some significant differences in anthocyanin levels. This was not surprising since the anthocyanins are more sensitive to environmental change (Stafford, 1990). Changes in anthocyanin levels occurring on ripening are also more dramatic than for the flavonols and thus slight variation in the time of harvest can have a significant effect. One of the 'Gala' genotypes, GS 109 ('Gala' x 'Splendour' cross), was significantly different from the others in flavonoid levels but was not significantly different from 'Splendour' and related genotypes (1/1, 1/21, 3/11) in flavonol levels. Three genotypes with 'Golden Delicious' parentage ('McKenzie', 4926 & 4629) were significantly different in total flavonol levels from the parent 'Golden Delicious'. However, they were not significantly different from each other and may possibly be closer to 'Red Dougherty' which is the other parent in all three genotypes. As with the other genotypes there were significant differences in anthocyanin levels.

Distribution of flavonoids in different apple tissues

There was a wide variation in the concentrations of the various flavonoids in different parts of the apple tree but the compounds present were common to most tissues. The flavonoid concentrations in the bark may have been underestimated since this tissue could not be ground as finely and all flavonoids may not have been extracted. Also, due to the different tissue type it is possible that the proanthocyanidins may have been bound to the tissue and not released by the extraction procedure. Although there were no major differences in the compounds present there were significant differences in the concentrations of the

various flavonoid groups, and small differences in the composition of the quercetin glycosides. There were also some differences in the composition of the unidentified compounds. Several other compounds were present in the flower, leaf and bark samples which had a similar retention time on the HPLC and similar UV spectra to phloridzin. These were probably other phloretin glycosides, possibly those that have been previously reported in apple skin, such as phloretin xylogalactoside and phloretin xyloglucoside (Williams, 1960; Oleszek *et al.*, 1988; Burda *et al.*, 1990).

Apart from phloridzin most other flavonoids present in all tissues had the 3′,4′ hydroxylation pattern (quercetin glycosides, cyanidin glycosides, (-)-epicatechin). In all apple tissues, apart from the fruit, phloridzin accumulated in the highest concentrations. Phloridzin is derived from a branch point early on in the flavonoid biosynthetic pathway (Figure 2.10) which could indicate a control point possibly at the flavanone 3-hydroxylase (flavanone→DHK) leading to a pooling of the phloretin glycosides. This step is obviously not rate-limiting in fruit. It is interesting to note that in the fruit the phloridzin is almost entirely located in the seeds where there are few other flavonoids present. The genes for the various enzymes involved in the flavonoid biosynthetic pathway appear to be the same in all plant tissues. However, there was variation in concentrations of the different flavonoid groups, this may be the result of control of the specific genes or the levels of some other controlling factors.

It may be of some biological significance that phloridzin is found in all other parts of the apple apart from the fruit flesh and skin. Phloridzin is well known to have physiological activity in the mammalian body causing glycosuria by interfering with the tubular reabsorption of glucose in the kidney and by inhibiting absorption of glucose from the small intestine (Harborne, 1967). Since phloridzin is bitter tasting (Lea & Timberlake, 1974) it may well prevent the rest of the plant from being damaged yet allowing for fruit to be eaten for seed dispersal. Phloridzin is also known to have other functions in the plant. Quinone oxidation and polymerisation products of phloretin by polyphenolase may be involved in the defence mechanism of apple leaves against the scab fungus *Venturia inaequalis* (Raa & Overeem, 1968).

2.5 Conclusions

The range of flavonoids detected in New Zealand-grown apple cultivars was similar to those reported in previous worldwide studies with three main groups of flavonoids present: anthocyanins, flavonols and proanthocyanidins. The major anthocyanin was cyanidin-3-galactoside with traces of other cyanidin glycosides, the identity of which still have to be determined conclusively. Flavonols were predominantly quercetin glycosides with only traces of kaempferol glycosides. Composition of the glycosides was similar to those reported previously, although more were reported together in a single cultivar than in previous studies. As with the anthocyanins the galactoside was generally the predominant glycoside. The only new report was the presence of quercetin-3-arabinopyranoside in apple skins although this was only a minor component. (-)-Epicatechin was the predominant proanthocyanidin together with its dimer procyanidin B2. The only proanthocyanidin detected and identified and not reported previously in apple skin was procyanidin B5. Further work is required to identify some minor proanthocyanidin components that have not been reported previously. Small quantities of the dihydrochalcone phloridzin were also detected in some apples.

Differences between apple genotypes and species in the flavonoid composition were quantitative and not qualitative. Red apple types differed only in the presence of anthocyanin with the composition of the other flavonoids (flavonols and proanthocyanidins) being the same. The same was true for the different apple tissue types with no major qualitative differences detected, although there were more significant differences in the quantities of the various flavonoid groups.

Thus apples showed a predominance of 3′,4′-dihydroxylated flavonoids with only traces of 3′- (e.g. kaempferol glycosides and phloridzin) and 3′,4′,5′-hydroxylated compounds (e.g. (+)-gallocatechin) indicating a close biosynthetic relationship. The control of flavonoid biosynthesis is at the level of production and in red cultivars the control of anthocyanin biosynthesis is at the final step(s) of biosynthesis.

Section 3 - FACTORS AFFECTING APPLE FRUIT COLOUR

3.1 Introduction

3.1.1 Pigments in fruit

As discussed previously there are several groups of pigments that are responsible for fruit colour: (1) the phenolic pigments located in the vacuoles, which include the anthocyanins; (2) the plastid pigments, chlorophyll and carotenoids; (3) brown pigments from the oxidation and polymerization of phenolic compounds. Only the first two groups of pigments are discussed here. Brown pigments do not generally play an important role in the skin colour of apples although browning is an important quality characteristic due to its appearance on processing and wounding of the fruit.

The flavonoids are the major group of phenolics involved in colour in fruits although other compounds, such as chlorogenic acid, may have a role in copigmentation. The most important group of flavonoids, in terms of colour, are the anthocyanins which give their colour to red, blue, mauve, and black fruits (Macheix *et al.*, 1990). Very significant changes take place during the life of the fruit, one of the most characteristic being the accumulation of anthocyanins during maturation (Macheix *et al.*, 1990); this is investigated in Section Four. Flavonol glycosides are not thought to contribute significantly to the yellow colour of fruit, which is usually due to the presence of carotenoids (Brouillard, 1988). However, they may be important as they enhance anthocyanin colouration by copigmentation (Asen *et al.*, 1971a & b; Chen & Hrazdina, 1981; Brouillard, 1983). Flavan-3-ols , which may be present as monomers or in condensed forms (proanthocyanidins), also occur in many fruits and may play a similar role (Macheix *et al.*, 1990). Those flavonoids, such as chalcones and aurones, which are known to contribute to the yellow colouration of some flowers (Brouillard, 1988) are not present in apples.

The final colour of the fruit is determined by a number of genetic controls but is also influenced by environmental factors. The anthocyanins, responsible for the red colour, may either be controlled by genes and factors that have a direct influence on them and others which modify our perception of the colour. These include: (1) genes controlling the nature and concentration of the anthocyanin; (2) genes and factors controlling *in vivo* stabilization of the colour by copigmentation and metal chelation; (3) genes controlling pigment distribution; (4) genes and factors controlling concentration of the plastid pigments, chlorophyll and carotenoids; (5) environmental influences such as light and temperature.

There has been no full study of all the pigments present in different apple varieties and relating differences in composition and concentration to variation in fruit colour. This was the objective of this study.

3.1.2 The anthocyanins and fruit colour

In flowers five major factors are known to influence anthocyanin colouration: (1) nature of the anthocyanins, particularly the extent of hydroxylation of the B-ring; (2) concentration of the anthocyanin; (3) pH of the cell sap; (4) the molar ratio of anthocyanin to copigment; and (5) metal chelation. With regard to fruit colour some of these areas such as copigmentation are not as well understood and there is a need for further investigation of these. There are also a number of other factors that may be involved, that influence our perception of the red colour, such as distribution of the anthocyanin pigment, surface texture and the presence or absence of waxy layers.

Nature of the anthocyanins

Comparative study of different species or different cultivars of various red fruits has revealed very large variations in the nature and levels of anthocyanins. The anthocyanins pelargonidin, cyanidin and delphinidin produce scarlet, crimson and blue-mauve shades respectively with λ_{max} of 520, 535 and 546 nm in 0.01% HCl in methanol (Gross, 1987). Thus, depending on the amount and types of anthocyanin present, a range of red hues of varying intensities can be observed. These differences in hue are usually due to the qualitative differences but as was observed earlier (Section Two) in apples the anthocyanin composition was the same for all genotypes and species examined. Cyanidin was the only aglycone present and even the composition of the glycosides was consistent with cyanidin-3-galactoside being predominant in all genotypes. Taking this factor alone into consideration it would be expected that all apples would exhibit various intensities of crimson colouration. However, a much wider range of red hues are observed in apple fruit from orange-reds to dark purple-reds. Thus, we must take into account other factors, which include the influence of other pigments such as chlorophyll and carotenoids, copigmentation of the anthocyanins with other flavonoids and distribution of the pigments.

Anthocyanin concentration

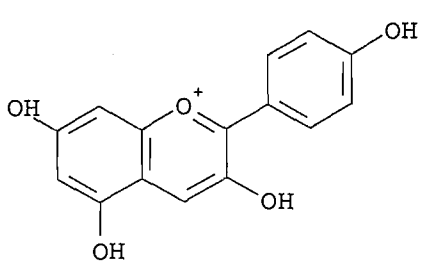
In apples large quantitative differences in the anthocyanin concentrations were observed (Section Two) and these could explain some of the variation seen in the intensity of fruit colour but this does not explain the differences in hue. The minimum energy requirement for anthocyanin synthesis has been determined and it was found that it varied considerably with cultivar and also during the season (Proctor, 1974). Anthocyanin concentration is influenced by a number of factors in addition to endogenous control. External factors also play a major role in fruit colour by acting on the rate of accumulation or degradation of anthocyanins and hence influence the final concentration of the pigments in the fruit (Walter, 1967). Other flavonoids may also be affected by these external factors but to what extent has not been as clearly determined as the anthocyanins. It has been noted that plant tissues containing high levels of anthocyanins can appear bluish because of the high absorbance of their vacuoles and their light-scattering effect (Chen & Hrazdina, 1981).

3.1.3 Factors in the expression of colour

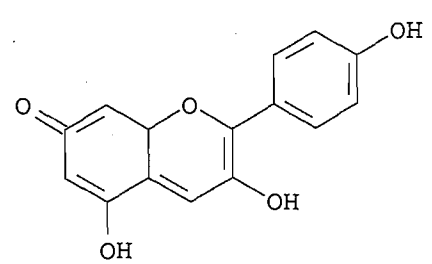
In addition to the nature of the anthocyanin the colour observed also depends on the immediate physiochemical environment of the molecule, which may stabilize and augment the coloured anthocyanins (Timberlake, 1981). These factors include the pH, the presence of other phenolic compounds and metal ions, with alteration of these factors resulting in a change of colour.

3.1.3.1 pH

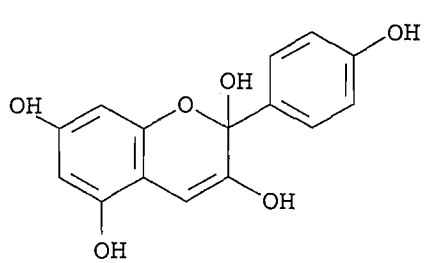
Many colour changes take place in the anthocyanins in aqueous solutions with changes in pH (Brouillard, 1988). This is because the flavylium cation is highly reactive with all three species of water, H^+, OH^- and H_2O. It has been well documented (Chen & Hrazdina, 1981 & 1982; Brouillard, 1982 & 1983) that anthocyanins can exist in four different forms (Figure 3.1) whose pH-related interconversions have been studied extensively: neutral and ionized quinonoidal A bases (blue or purple), flavylium cation AH^+(red or orange), pseudobase or carbinol B (colourless), and chalcone (yellow). Anthocyanins in an aqueous medium at 25°C and slight acidity, that is close to the natural vacuolar environment, transform to colourless forms (Macheix *et al.*, 1990). Anthocyanins only show intense colouration over a very limited pH range, from 1 to 3. An increase in pH causes reversible structural transformation with a loss



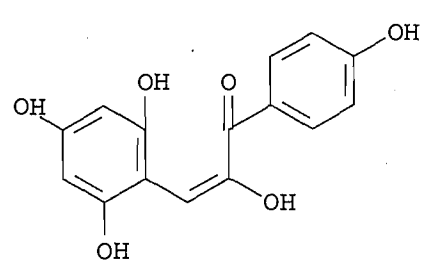
anthocyanidin flavylium cation
(pH 1-3; red)



neutral & ionized quinonoidal base
(blue or purple)



pseudobase
(pH 4-6; colourless)



chalcone
(pH>7; yellow)

Figure 3.1: The structural transformations of anthocyanins in aqueous solutions at room temperature at varying pH (from Brouillard, 1982).

of red colour. In the pH range from 4 to 6.5 transformation to the purple quinonoidal base occurs followed by the carbinol base with a progressive disappearance of the colour. Ionized quinonoidal bases also appear around neutrality and at higher pH ring fission produces a yellow chalcone. This chalcone is unstable and disappears as the pH increases further (Macheix *et al.*, 1990).

Thus pH not only exerts a profound influence on the colour of the anthocyanins but it also affects their stability (Markakis, 1982). Anthocyanins are more stable in acidic solutions than neutral or alkaline.

3.1.3.2 *In vivo* stabilization of colour

Under the slightly acidic aqueous conditions of the apple cell vacuole it would be expected that the anthocyanins would lose their colour. If colouration is to occur there must be a protection phenomenon which permits the stabilization of the anthocyanin in the coloured form of the flavylium cation. Recent research with a variety of plants has indicated that there are at least four ways in which the anthocyanins are stabilised *in vivo* (Figure 3.2). These involve self-association, intramolecular copigmentation, intermolecular copigmentation and metal complexing (Harborne, 1988). A fifth factor, malonylation, may also be involved but to what extent this has an effect *in vivo* is uncertain (Saito *et al.*, 1985).

Self-association

Self-association of anthocyanins in solution was first postulated by Asen *et al.* (1972) to explain why the absorbance of a cyanidin solution deviated from the Beer-Lambert law with increasing concentration. It appears that vertical stacking occurs in anthocyanins particularly with sugars at both the 3- and 5-positions which protects them from hydrolytic attack (Harborne, 1988). Above a certain critical concentration (possibly about 10^{-2} M) the flavylium cations are known to be nearly planar and to form molecular aggregates, but the significance of this *in vivo* is not yet known.

Although self-association is not well understood it appears that a small loss in pigment concentration can result in a proportionately great loss of colour, especially at high anthocyanin concentrations (Mazza & Miniati, 1993). Self-association has been reported to occur in certain fruits such as grape when high anthocyanin concentrations occur (Moskowitz & Hrazdina, 1981). There may also be competition between self-association and copigmentation (Scheffeldt & Hrazdina, 1978). However it is difficult to separate the two factors to determine how each may be contributing to the colour (Harborne, 1988).

Copigmentation

Copigmentation is an explanation for the intense and infinite colour variations that occur in plant tissues in a pH range where anthocyanins alone are virtually colourless (Asen *et al.*, 1972). Copigmentation involves either interaction between the anthocyanin molecules themselves (intramolecular copigmentation) or by the formation of complexes with other colourless phenolic molecules which play

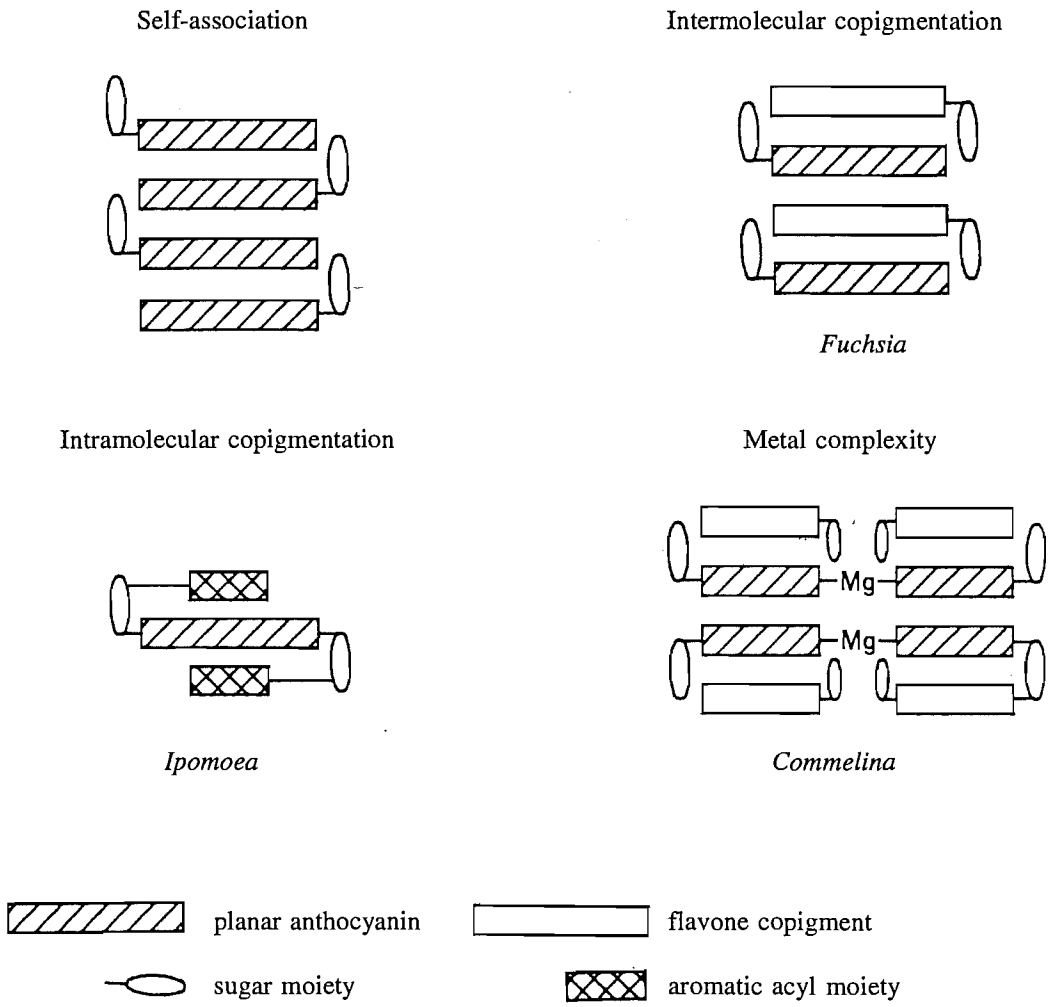


Figure 3.2: Mechanisms of anthocyanin stabilization *in vivo* (from Harborne, 1988).

the role of copigment (intermolecular copigmentation). Intramolecular copigmentation is responsible for the colour stability of anthocyanins containing two or more aromatic acyl groups. Since there are no such anthocyanins in apples this is not a factor in influencing colour.

Intermolecular copigmentation of anthocyanins with other flavonoids and related compounds produces an increase in colour intensity (hyperchromic effect) and a shift in the wavelength of maximum absorbance towards higher wavelengths (bathochromic shift). This results in purple and blue colours that could not normally be achieved with the anthocyanin alone (Asen *et al.*, 1972; Scheffeldt & Hrazdina, 1978; Osawa, 1982; Mazza & Brouillard, 1990). This type of copigmentation also aids in stabilization of the coloured form (Macheix *et al.*, 1990). Copigments may be a number of different substances including other flavonoids, polyphenols, alkaloids, amino acids, organic acids and the anthocyanins

themselves. Flavonoids are common copigments since they often occur together with the anthocyanins in the vacuole. In contrast to metal chelation and intramolecular copigmentation, intermolecular copigmentation occurs with the glycosides of all six common anthocyanins, including cyanidin.

The copigmentation effect has been shown to be a molecular interaction occurring between the anthocyanin coloured form and the copigment (Brouillard *et al.*, 1989; Mazza & Brouillard, 1990). The main role of the copigment is that of controlling the extent of the hydration reaction between the flavylium cation and the colourless pseudobase (Mazza & Miniati, 1993). The intensity of the copigmentation effect is dependent on a number of factors in particular the type and concentration of the anthocyanin and copigment, and the pH (Macheix *et al.*, 1990). One of the most effective copigments are the flavonols which are abundant in apples. Colour intensification by copigments increases with an increasing ratio of copigment to anthocyanin and also increasing anthocyanin concentration (Mazza & Brouillard, 1990). The effect is weak when the two concentrations are low and when the anthocyanin concentration is weak (Asen *et al.*, 1972; Asen, 1976), e.g. less than 3.5×10^{-5} M for cyanidin-3-glucoside (Macheix *et al.*, 1990). Copigmentation occurs at pH 1 to near neutral with the maximum effect at about pH 3.5 (Brouillard *et al.*, 1989). Change in pH results in a change of the shade of colour, which is often noted as in the blueing of aging flowers (Harborne, 1988).

With flowers there has been extensive work on the relationship between petal colour, the type of anthocyanin and copigmentation (Asen *et al.* 1971a & b, 1972, 1975 & 1986; Goto & Kondo, 1991). Copigmentation has been shown to offer a suitable explanation for the colour of many flowers including 'Prof. Blaauw' iris, 'Red Wing' azalea, and 'Better Times' rose (Asen *et al.*, 1972). However the contribution of the copigmentation phenomena to the appearance of the fruit is not yet as well researched, yet many fruit have an abundance of flavonols and proanthocyanidins suitable for copigmentation. The fruits of different *Ilex* species suggested that copigmentation occurred in fruits that contained flavonols and anthocyanins since these showed a bathochromic shift in the λ_{max} over fruit containing only anthocyanins and not flavonols (Ishikura, 1975). However, in the grape (*Vitis vinifera* L.) cv. De Chaunac the copigmentation effect was shown to be very weak or nil which was probably because the anthocyanin concentrations were up to 97 mM whereas the total flavonol glycoside concentration was only in the order of 0.5 mM (Moskowitz & Hrazdina, 1981). It has been seen that significant effects were only found with equimolar or higher flavonol glycoside contents (Asen *et al.*, 1972). Thus, when studying the red colour of fruit, it is important to look at the concentration and composition of the copigments as well as the anthocyanins themselves. Studies need to be widened to other fruit, such as apples, and to include other molecules that may be involved in copigmentation such as catechin and proanthocyanidins. The ratios of flavonols and proanthocyanidins to anthocyanins were determined for a range of different apple genotypes and species in an attempt to relate pigment composition and copigmentation to variation in fruit colour.

Metal complexing

Some anthocyanins readily form metal complexes in solution resulting in a shift of the colour to the blue region of the spectrum and this has led to the idea that such complexing may lead to the stabilization of anthocyanins *in vivo* (Harborne, 1988). However due to the problems of isolating these complexes there is still much debate about their influence. In two plant species, *Commelina communis* and *Hydrangea macrophylla*, the evidence of metal complexing is reasonably secure although other stabilizing features are present and it has not been determined if the metal complexing is essential for stabilization. Metallic, ions like aluminium (Al^{3+}) and iron (Fe^{3+}), have been shown to interact in many species and magnesium seems to be involved in the pigment complex commelinin from blue flowers of *Commelina communis* (Osawa, 1982). The blueing of the red sepals of *Hydrangea macrophylla* has been shown to be the result of complexing of the anthocyanin with aluminium (Harborne, 1988). Takeda *et al.* (1985) also found that 3-caffeoylquinic acid was required as a copigment for the blueing process. In other cases it has been noted that some compounds (chlorogenic acid, flavonols) alone do not have a measurable effect on the colour or stability of the anthocyanin but in the presence of metals (e.g. Al^{3+}) they form stable complexes (Jurd & Asen, 1966). It is possible that Al^{3+} and other metallic ions, that are often abundant in fruit, may play an important role in the blue colouration of some fruit but the results to date are very fragmentary (Macheix *et al.*, 1990).

Goto *et al.* (1976) reported that anthocyanin quinonoidal forms and flavylium ions are strongly oxidised and do not form colourless pseudobases by hydration when dissolved in concentrated aqueous solutions of neutral salts such as magnesium chloride and sodium chloride. It has been suggested that colour stabilization in NaCl may be due to promotion of self-association and stabilization of $MgCl_2$ to a reduction in the concentration of free water by hydration of magnesium ions (Mazza & Miniati, 1993).

3.1.3.3 Pigment distribution

Another factor that may have some influence on the intensity of the red colour of the apple skin is the distribution of anthocyanins in the skin cells. The distribution of the anthocyanins in the fruit is under genetic control and the consequences with regard to colouration can be important (Macheix *et al.*, 1990). For example in holly (*Ilex rotunda* and *crenata*) the number of coloured cell layers plays a large part in determining the intensity of fruit colour (Ishikura, 1975). Red fruit skin of *I. rotunda* is composed of a single layer of light red cells, whereas the black fruit skin of *I. crenata* is composed of several layers of deep red cells.

Work carried out at this research institute (Lancaster *et al.*, 1994) revealed a non-uniform distribution of anthocyanin between apple fruit epidermal cells and this was the same for all genotypes studied. Very dark red cells were adjacent to pale cells but within a cell the vacuoles were uniform in appearance (Plate 3.1). The reason for these differences in colour intensity are unknown as there is no pattern to the distribution; it does not suggest a cytological or developmental mechanism. Darker coloured

Plate 3.1: Anthocyanin distribution in apple skin (tangential sections)

(a) Granny Smith

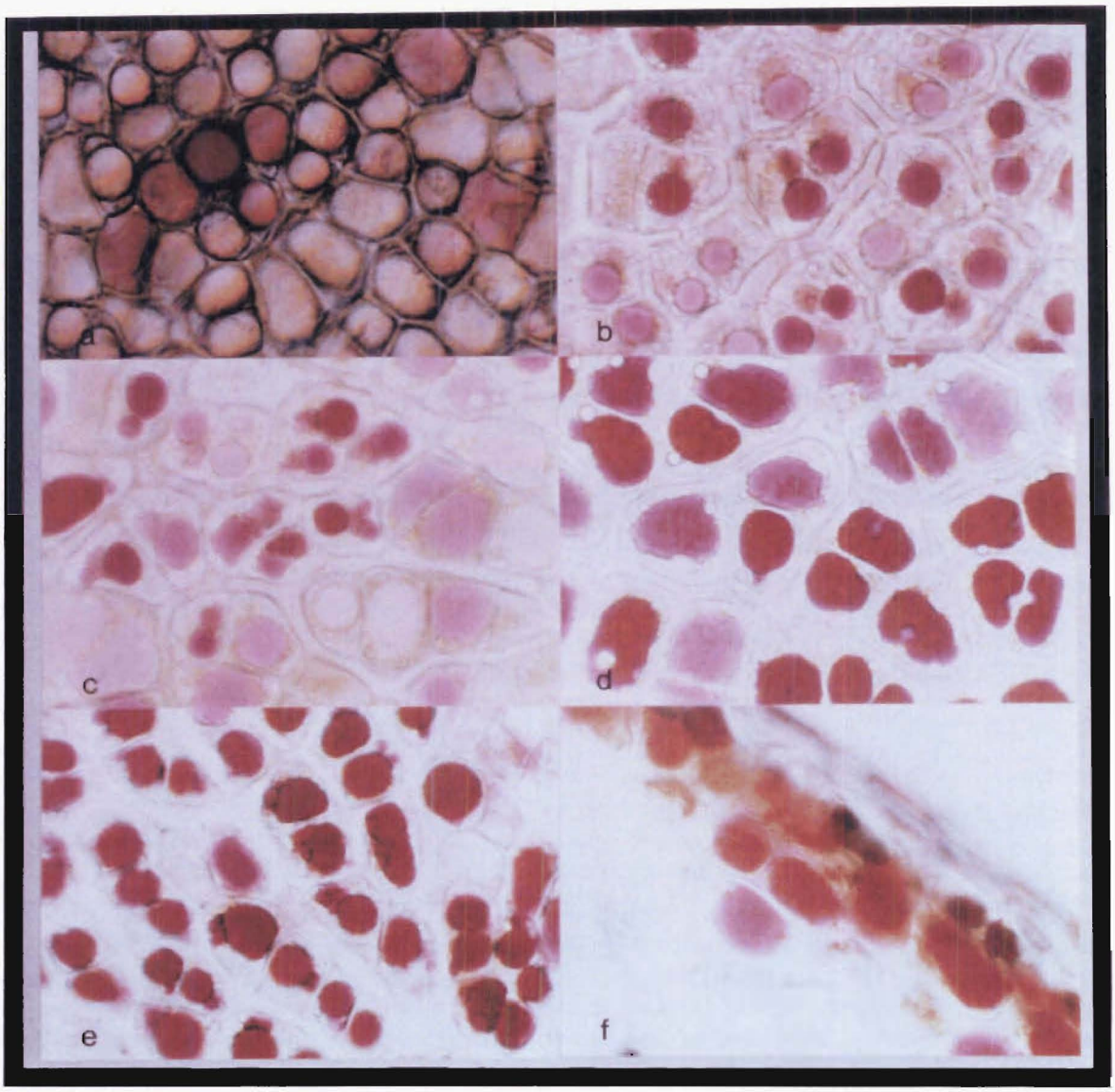
(b) 3827

(c) 4926

(d) Regal Gala

(e) Oregon Red Delicious

(f) Oregon Red Delicious (transverse section)


a
b
c
d
e
f

genotypes had a greater proportion of cells with higher absorbances at 550 nm and larger vacuoles, although all genotypes examined showed some cells without anthocyanins in their vacuoles. One anomaly was observed; 'Oregon Red Delicious' was much darker in appearance than 'Regal Gala' although 'Regal Gala' had a higher proportion of higher absorbance cells. However in 'Oregon' the anthocyanin was present in up to three layers of cells in the epidermis and the other genotypes, including 'Regal Gala', usually only had a single layer. There were also large differences between genotypes in the size of the vacuoles within the cells although the cell size was uniform. In the darker red genotypes, 'Regal Gala' and 'Oregon Red Delicious', the vacuoles represented about 50% of the surface area and this was also similar for 'Granny Smith'. However in the paler red apples, genotypes 3827 and 4926, had about 20 and 30% respectively of the cell area taken up by the vacuole. Thus the later two genotypes do not have the same potential for reddening. The distribution of the anthocyanin in the skin and variation in vacuolar size only accounts for variation in the intensity of the red colour but not for the differences in the hue.

3.1.3.4 Other factors

In addition to these factors discussed above the intensity and stability of the colour of the anthocyanin are influenced by other factors such as light, temperature, oxygen, sugars, sulfur dioxide and amino acids. These factors have been reviewed in general (Markakis, 1982; Gross, 1987) and with specific regard to apples (Walter, 1967; Saure, 1990). In apple skin sugars (fructose, glucose, lactose, maltose and sucrose) are known to stimulate anthocyanin synthesis (Macheix *et al.*, 1990). Calcium carbonate increases red colour formation in 'McIntosh' apples, and an additive effect on calcium carbonate and sugars (sucrose and galactose) on anthocyanin synthesis has been shown (Smock, 1966 & 1969). Thus, Ca^{2+} ions appear to enhance synthesis of anthocyanins in disks of apple peel, but the effects depend on the stage of maturity of the fruit and on the form in which the Ca^{2+} is supplied (Vestrheim, 1970). Generally these factors affect the intensity of fruit colour rather than alteration of the hue although they may alter this indirectly by changing copigmentation or self-association factors.

3.1.4 Influence of plastid pigments

In flowers the yellow and green plastid pigments are well-known to influence the appearance of the petals. Yellow plastid pigments in the presence of red vacuolar pigments give an orange-red or bronze phenotype (Paech, 1955). The orange colour of some chrysanthemums (*Dendranthema grandiflora* Tzvelev.) was found to be the result of a mosaic-like distribution of red vacuolar pigmentation over yellow plastid pigmentation (Teynor *et al.*, 1989a). The relative significance of plastid pigmentation, together with the anthocyanins, to the final appearance of the fruit has not received as much study. Although these compounds decrease during fruit development there are still significant amounts present in many mature fruit to influence colour. Most studies of the plastid pigments have been focused on cultivars where these

are the predominant pigment such as 'Golden Delicious' and 'Granny Smith'.

Chlorophyll

Green plants owe their colour to the most important plant pigments, the chlorophylls, which participate in the fundamental life process of photosynthesis, the transformation of light energy into chemical energy (Gross, 1987). All green plants contain chlorophylls *a* and *b*. The chlorophylls are porphyrins, which have a basic skeleton of a tetrapyrrole ring with a long hydrophobic side chain attached. The chlorophylls are green in colour because they absorb strongly in the blue and red regions of the spectrum (Holt, 1965).

Chlorophyll can be determined in plant extracts using various methods based on spectrophotometry or spectrofluorometry, but owing to their lability precautions must be taken to avoid their alteration (Gross, 1987). All manipulations must be carried out rapidly, at low temperatures, and in dim light to avoid photobleaching, heating, oxidation, and to prevent chlorophyllase action. Solvents like acetone are used which break the chlorophyll-protein complex non-covalent linkage and extract the pigments quantitatively. Undiluted solvents also block the chlorophyllase activity (Gross, 1987).

There has been little study on the variation in chlorophyll levels in apples. Most of the work done has been centred around developmental or storage changes in green genotypes such as 'Granny Smith' (Mussini *et al.*, 1985). Data on chlorophyll levels in red genotypes and the contribution it makes to their overall appearance is lacking and thus was investigated in this study.

Carotenoids

The yellow, orange and red colours of many fruits are due to the presence of carotenoids. Carotenoids are among the most widespread and important natural pigments and together with chlorophylls they are found in all organisms capable of photosynthesis (Gross, 1987). The colour of carotenoids is due to the presence of a series of conjugated double bonds present in the molecule (Goodwin & Britton, 1988).

The carotenoids which accumulate in apple skin have been extensively investigated (Gross, 1987). The composition of the individual carotenoids has been determined for a number of apple cultivars (Gross *et al.*, 1978) and this was not examined here, only the total carotenoids were measured by a simple spectrophotometric assay. In one early report the carotenoids in both peel and pulp were determined and found to be 37μg g^{-1} FW in peel and 13μg g^{-1} FW in pulp of 'Golden Delicious' and 59μg g^{-1} FW in peel and 16μg g^{-1} FW in the pulp of 'Grimes Golden'. (Workman, 1963). The carotenoid pattern of apples grown in England was found to be different. Total carotenoid content (56 and 85μg g^{-1} FW) was two to three fold higher in peel than pulp (Valadon & Mummery, 1967). In a more recent study by Gross *et al.* (1978) the total carotenoid content of the peel of Golden Delicious apples was approximately 20μg g^{-1} FW. Most of this work has concentrated on yellow fruit. Although carotenoids are still present in most red apple fruit there is little data on this and the modifying effect that carotenoids may have on the colour of the fruit.

3.1.5 Measurement of colour

The first appraisal of fruit colour is provided by the eye but it is difficult to estimate visually the area and intensity of red colour because some apples are striped, others are blushed and the intensity of each may vary considerably. Although visual appraisal of fruit colour is important it is necessary to use objective measurements of colour for quantitative comparison. There are two types of methods that can be used for colour measurement: nondestructive and destructive (Macheix *et al.*, 1990).

Nondestructive methods

Nondestructive methods are based on the determination of characteristics of light transmitted or reflected by the fruit (Macheix *et al.*, 1990) and this can be done in a number of ways. Lott (1944) measured skin colour with a recording spectrophotometer but the data was very hard to interpret and the equipment is expensive. Tristimulus colorimeters have been used for a variety of agricultural products successfully for many years; these include tomatoes, carnations, roses, corn, peas, turnips and potatoes (Francis, 1952). They have also been used, with varying success, to measure the skin colour of apples (Francis, 1952; Crassweller *et al.*, 1984; Singha *et al.*, 1991a & b).

A colour is characterized by three attributes: hue, lightness and saturation which, when translated into a generally accepted system, become hue, value and chroma (Gross, 1987). The relationship of these attributes can be explained by what is known as a colour solid which is a graphical representation of the

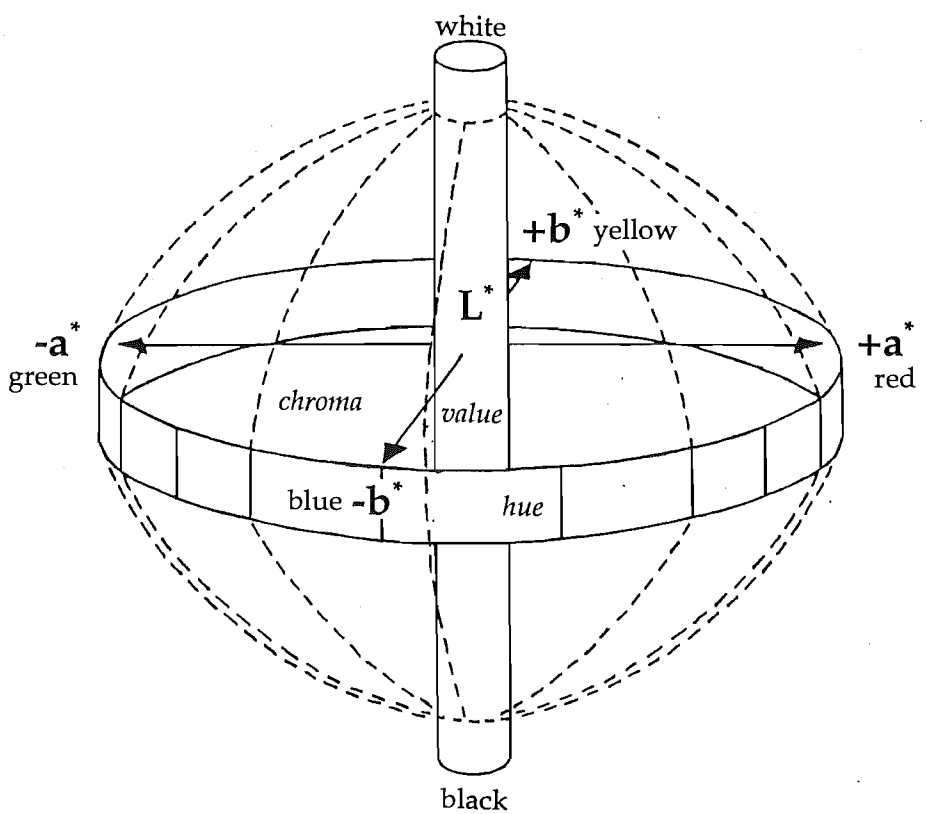


Figure 3.3: Solid $L^*a^*b^*$ colour chart

three attributes of colour (Figure 3.3). This shows the commonly used CIE (Commission Internationale de l'Eclairage) $L^*a^*b^*$ colour space system (abbreviated CIELAB), and which is the system that was used in this study. There are also a number of other systems that can be used including Munsell scales, Adams-Nickerson scales, and Y *x y* values (Hunter & Harold,1987). Hue is the colour and using this $L^*a^*b^*$ system is represented by a^* and b^*. Measurements of a^* are negative for green and positive for red and b^* is negative for blue and positive for yellow. Chroma is the degree of saturation and is equal to $\sqrt{(a^{*2}+b^{*2})}$. Value is the lightness and is represented by L^*, being small for dark colours and large for light colours. For fruits the a^*/b^* ratio is usually computed. This ratio is negative for green fruits, approximately 0 for yellow fruits and positive for orange or red fruits (Gross, 1987). Francis (1952) recognized pitfalls inherent in expression of hue in terms of a^*/b^* for apples which cover a wide range of colours. Thus the adoption of an angular function, $\tan^{-1} a^*/b^*$ which represents the angle between the constant hue line and the vertical axis, was suggested. However, this changed the well-understood coordinate system in order to maintain a positive relation between increasing redness and the numerically expressed hue function (Little, 1975). The hue angle concept is now widely acknowledged but conceptually the b^*/a^* ratio is the expression of choice (Little, 1975). For the purpose of this study both values were examined.

Francis (1952) determined that Hunter Rd, a, b and tan-1 a/b values could be used as an index of apple skin colour and maturity. However only one cultivar, 'McIntosh', was examined. Likewise, Singha *et al.* (1991a) studied the relationship between anthocyanin concentration and chromaticity values and showed that the values allowed nondestructive estimation of the anthocyanin content *in situ*. This study was also limited in that only strains of 'Delicious' were examined. There have been no studies relating $L^*a^*b^*$ values, and the calculated colour components of hue and chroma, to the pigment composition of different apple cultivars which was the purpose of this study.

Destructive methods

Destructive methods involve the extraction and assay of the various pigments concerned with fruit colour. One of the difficulties in interpretation resides in the complex relations that may exist between the overall colour of the fruit, as perceived by the eye, and the quantitative analysis. Problems arise due to the extraction which may lead to modification of colour, in particular of the anthocyanins. These can be due to: (1) combining of different shades of tissue such as anthocyanin rich with tissues that contain none; (2) mixing of vacuolar contents with other cell compartments which have different physicochemical conditions; (3) modification of the environment such as pH; (4) dissociation of complexes due to copigmentation or with metals; (5) instability and degradation of pigments due to enzymes and external factors (temperature, light); (6) disappearance of interference between light absorption by anthocyanins and other pigments (Macheix *et al.*, 1990). Some of these problems can be minimized by taking care with methods, however some changes cannot be avoided. Thus, it is necessary to review relations between data obtained by non-destructive methods and the analysis of pigments.

3.2 Materials and methods

Materials

Plant material

Apples were collected at maturity from several different orchards at the end of the 1989/90 and 1990/91 seasons (for descriptions and sources of genotypes and species see Appendix A). Usually three apples for each genotype or species, being representative of the colour of that type, were taken. Apples were kept in a cool room (+4°C) prior to experimentation but colour measurements were taken and skin samples prepared as soon as possible (usually within a week) to prevent changes occurring during storage.

Flavonoid standards

Flavonoid standards were as listed in Section 2.2.

Methods

Handling of fruit for pigment extraction

Directly after nondestructive colour measurements the peel was carefully removed from the whole of each fruit taking care to remove any underlying flesh with a scalpel. It was snap frozen in liquid nitrogen and then stored at -20°C until the pigments could be extracted and assayed. Half the peel from each fruit was used for flavonoid estimation and the other half for assay of chlorophyll and total carotenoids. A few species were crabapple types (*Malus micromalis*, *Malus pumila niedwitzkiana* and *Malus soulardi*) and had very small fruit so these samples were the combination of several fruit. Samples collected in 1989/90 season were not analyzed for proanthocyanidins.

Quantification of the flavonoids

Data obtained in Section Two on the levels of the flavonoid groups for the different genotypes and species was used for colour analysis.

Calculation of molar ratios of the flavonoids

The average percentage dry matter of the apple skin was calculated at approximately 30%, thus for the purpose of calculations one gram of tissue was equivalent to 0.7 ml of solution (which is contained largely in the vacuole where the flavonoids are also located). The molecular weights used were anthocyanins 484, flavonols 464, and proanthocyanins 464 and were calculated based on the average composition of the individual components.

Extraction and estimation of chlorophylls

The methods used for determination of chlorophyll and total carotenoid content were essentially the same as those used by Knee (1972). Where possible the extracts for both these assays were kept cold and in the dark to minimize any pigment degradation. The apple peel tissue (0.5-1.0 g) was ground to a fine powder in a mortar and pestle using liquid nitrogen and then extracted with 15.0 ml of cold acetone. The residue was then re-extracted with 5.0 ml aliquots of 80% acetone until clear (usually twice). If necessary the combined extracts were made up to 30.0 ml with 80% acetone and were clarified by centrifugation, at 5,000 g for 10 min. The absorbance of these acetone extracts was measured at 645, 652 and 663 nm and a reading was also taken at 700 nm to correct for any turbidity. Chlorophyll content was calculated from these figures according to Holden (1965) using the following equations:

$$\text{Chlorophyll } a \text{ (mg g}^{-1}\text{ FW)} = \frac{12.3D_{663} - 0.86D_{645} \times V}{d \times 1000 \times W}$$

$$\text{Chlorophyll } b \text{ (mg g}^{-1}\text{ FW)} = \frac{19.3D_{645} - 3.6D_{663} \times V}{d \times 1000 \times W}$$

where V = volume in ml, d = length of light path in cm, W = fresh weight in g.

To check on the presence of pheophytin and other breakdown products the total chlorophyll concentration was also calculated from the measurement made at 652 nm:

$$\text{Total Chlorophyll (mg g}^{-1}\text{ FW)} = \frac{27.8D_{652} \times V}{d \times 1000 \times W}$$

Total carotenoid estimation

Total carotenoids were determined by taking the crude acetone extract, prepared as above, and removing the chlorophylls by saponifying the extract as follows. An aliquot (10.0 ml) of the acetone extract was taken and 10.0 ml of petroleum ether (fraction boiling between 40 and 60°C) and 3.0 ml 50% saturated aqueous ammonium sulphate were added. The upper phase was washed twice with 3.0 ml aliquots of ammonium sulphate. 1.0 ml of 25% (w/v) potassium hydroxide in methanol was added and the mixture swirled occasionally for 15 min. The upper phase was washed with successive 5.0 ml portions of water until it was optically clear (usually three or four washes). The petroleum ether solution was dried over anhydrous sodium sulphate for at least one hour before reading the absorbance at 446 nm. A reading was also taken at 550 nm to correct for any turbidity. For the basis of calculation of the total carotenoids the extinction coefficient used for this crude mixture was that of ß-carotene, $E^{1\%}_{1cm} = 2500$ (Goodwin, 1955).

$$\text{Total Carotenoids (mg g}^{-1}\text{ FW)} = \frac{D_{446} \times 10 \times V}{d \times E^{1\%}_{1cm} \times W}$$

Nondestructive colour measurements

Four zones representing a range of colour shades and intensities were measured on each fruit which covered both top and bottom of the fruit and sun and shade sides. The fruit were carefully wiped before measurement to remove any surface grime but were not polished to a shine. Colour measurements were made using the 8 mm diameter head of a Minolta Chromameter II, portable tristimulus colour analyzer. Measurements were expressed in CIELAB colour space coordinates. The meter was calibrated using the manufacturer's standard white tile. The colour attributes of chroma [$\sqrt{(a^{*2}+b^{*2})}$], hue (b^*/a^*) and hue angle ($\tan^{-1} b^*/a^*$) were calculated from this data, noting that for the hue angle where $a^*<0$ and $b^*>0$ the hue angle = $180+\tan^{-1} b^*/a^*$.

Statistical analysis

Concentrations of individual pigment groups were calculated and $L^*a^*b^*$ colour space coordinates taken for each apple and the average calculated. ANOVA (using the method for samples of unequal sizes) was used to establish differences between genotypes for all data. All LSD's are given at the 5% significance level.

3.3 Results

Pigment levels

(a) Flavonoids

As discussed in Section Two (and for details refer to Appendices B and C) there were only minor differences in the composition of the individual flavonoids in the various genotypes and species examined and these were not the significant differences that would account for variation in colour. However, the levels of the main flavonoid groups (anthocyanins, flavonols, phloridzin and proanthocyanidins) varied considerably with significant differences between the various apple genotypes and species (Table 3.1). Anthocyanins were absent in non-red genotypes and species and in genotypes where they were present levels varied up to a maximum of 4.3 mg g^{-1} FW. In general, the anthocyanin concentration increased with a visual increase in red colour. Flavonols were present in all genotypes and species and the concentrations varied from 0.6 mg g^{-1} FW up to 10.1 mg g^{-1} FW but did not correlate with visual estimation of yellow colour. Those genotypes with the highest yellow colour, 'Golden Delicious' and *Malus sylvestris*, had very low levels of flavonols compared to 'Ikorokavka Alajah' which had a white background yet moderate flavonol levels. Phloridzin was only present in some apple genotypes and species with levels between 0 and 0.06 mg g^{-1} FW. Proanthocyanidins were also present in all the apples samples and the concentrations ranged from 1.3 to 6.2 mg g^{-1} FW. These last two classes of compounds are both colourless so cannot be directly correlated with any colour attribute.

In all but two genotypes flavonols were the predominant flavonoid group, in 'Red Cox' anthocyanins were higher than flavonols (proanthocyanidins were not measured) and in 'Oregon Red' proanthocyanidins were slightly higher than the flavonols, but not significantly. There was no apparent pattern to the division of the flavonoids into the different groups (i.e. anthocyanins, flavonols and proanthocyanidins). In some cases levels of all three groups were similar ('Oregon Red') but in other cases one group predominated such as flavonols in the genotype 4 last. There was no significant correlation between anthocyanin concentration and flavonol levels (R^2=0.015), or anthocyanin and proanthocyanidin concentration (R^2=0.14), or anthocyanin and total flavonoid (R^2=0.096).

(b) Chlorophyll and carotenoids

There was wide variation in both the levels of carotenoids and chlorophyll and there were significant differences between many of the genotypes and species (Table 3.1). It can be seen that carotenoids were present in all genotypes and species but the levels varied considerably. The lowest levels were 3.23 μg g^{-1} FW and 3.32 μg g^{-1} FW, in the genotypes 3/11 and 'Ikorokavka Alajah' respectively, up to a maximum of 29.48 μg g^{-1} FW and 33.48 μg g^{-1} FW in 'Lawfam' and 'Maling 9'. The other genotypes and species cover the whole range between these values with an average of 15.49 μg g^{-1} FW. The range of chlorophyll levels was even greater with many genotypes containing no chlorophyll at maturity, these were mainly 'Gala' genotypes, through to 'Lawfam' and 'Granny Smith' with levels of 155.80 μg g^{-1} FW

Table 3.1: Levels of the pigment groups in the skin of apple genotypes and species

Apple genotype or species	Pigment concentration (μg g^{-1} FW)					
	Carotenoids	Chlorophyll	Anthocyanins	Flavonols	Phloridzin	Proanthocyanidins
1/1	11.91	0.00	1392.98	6303.38	18.03	4443.62
1/21	3.23	0.00	233.34	7401.30	23.20	1556.57
3/11	5.28	28.51	372.76	6923.68	21.00	1913.10
3/16	16.96	0.00	178.91	4487.01	19.19	-
3827	9.55	15.53	554.47	4234.84	9.07	1771.29
4692	10.01	67.00	128.40	4820.50	0.00	-
4926	21.18	21.85	834.18	5696.06	8.27	2408.35
4/1	23.79	0.00	457.53	3690.92	0.00	-
4/7	14.70	0.00	295.38	2979.25	11.32	-
4/17	10.23	55.32	759.91	3647.98	14.27	1971.92
4 last	12.87	37.91	70.86	9558.78	30.57	1305.86
666	23.16	0.00	82.70	5108.89	0.00	-
92 Just	13.58	9.09	1582.37	3247.75	4.00	2851.72
'Braeburn'	10.47	45.90	124.35	6187.53	26.63	-
'Cox's Orange Pippin'	24.64	38.30	265.08	1486.29	0.00	-
'Fuji'[1]	20.24	97.90	0.00	8710.86	21.82	-
'Fuji'[2]	11.08	38.85	81.67	6224.78	9.30	-
'Golden Delicious'	6.48	34.88	0.00	1725.53	0.00	-

Table 3.1: *continued*

Apple genotype or species	Pigment concentration (μg g^{-1} FW)					
	Carotenoids	Chlorophyll	Anthocyanins	Flavonols	Phloridzin	Proanthocyanidins
'Granny Smith'[3]	21.69	170.80	0.00	3227.98	0.00	-
'Granny Smith'[4]	-	-	0.00	3565.57	17.40	2012.99
GS 109	9.68	52.10	1336.20	3150.17	0.00	-
GS 150[3]	9.70	12.00	978.23	5198.18	0.00	-
GS 150[4]	14.00	0.00	985.08	5664.56	11.50	2808.34
Hyde 5/22	14.68	0.00	1825.08	2585.22	0.00	-
Hyde 5/30	16.98	0.00	458.74	3773.53	11.97	-
'Ikorokavka Alajah'	3.32	0.00	557.89	4092.69	10.83	1843.66
J997[3]	12.64	0.00	610.56	4278.82	12.23	-
J997[4]	16.54	0.00	1442.57	5284.10	60.91	2031.15
'Lawfam'[3]	25.51	120.32	2352.69	9944.58	25.92	-
'Lawfam'[4]	29.48	155.80	1040.78	10145.74	37.46	1618.72
'McKenzie'	12.39	61.45	50.12	3963.39	0.00	-
'Maling 9'	33.48	32.42	0.00	1739.43	0.00	-
Malus micromalus	18.17	0.00	726.83	3917.24	0.00	-
Malus pumila niedzwetzkyana	28.14	0.00	1130.64	2054.85	0.00	-
Malus soulardii	23.61	14.70	13.06	901.49	0.00	-
Malus sylvestris	13.43	29.78	0.00	623.73	0.00	-

Table 3.1: *continued*

Apple genotype or species	Pigment concentration (μg g^{-1} FW)					
	Carotenoids	Chlorophyll	Anthocyanins	Flavonols	Phloridzin	Proanthocyanidins
'Oregon Red'[3]	7.58	32.46	3246.52	6205.64	0.00	-
'Oregon Red'[4]	9.68	52.10	4388.07	5914.48	11.05	6257.75
'Red Cox'	25.48	28.09	2732.89	2515.21	0.00	-
'Red Delicious'	12.18	41.61	936.86	3086.36	0.00	-
'Regal Gala'[3]	18.54	0.00	2018.13	2908.05	0.00	-
'Regal Gala'[4]	14.33	0.00	2042.27	4087.23	12.72	2973.34
'Seedling Gavin'	11.19	44.89	1181.02	5017.42	0.00	-
'Spartan'	19.42	30.40	1108.46	1691.22	0.00	-
'Splendour'[3]	8.99	44.39	885.69	4809.36	0.00	-
'Splendour'[4]	-	-	1177.71	4920.48	29.03	3558.43
'Sturmer'	15.04	84.67	56.23	8254.47	11.07	-
LSD 5%	5.2	23.9	483.7	2319.3	10.9	1322.3

[1] Sample collected from Havelock North Research Orchard
[2] Sample collected from Canterbury Orchard Systems
[3] Sample collected in 1990
[4] Sample collected in 1991
- Not quantified

and 170.80 μg g^{-1} FW. The average chlorophyll content was 33.31 μg g^{-1} FW. Both groups of plastid pigments varied in concentration independently as there was no correlation between carotenoid and chlorophyll levels (R^2=0.05). In most cases where chlorophyll was present it was at higher levels than the carotenoids (e.g. 'Granny Smith'). In other genotypes they were at similar levels (e.g. 4926) and sometimes the carotenoids were present in higher concentrations than chlorophyll (e.g. *Malus soulardii*). The plastid pigments also varied independently from the anthocyanins and total flavonoids. There were no correlations between carotenoids and anthocyanins (R^2=0.001), chlorophyll and anthocyanins (R^2=0.003), carotenoids and total flavonoids (R^2=0.06), or chlorophyll and total flavonoids (R^2=0.003).

Copigmentation

The molar concentrations of the total anthocyanins, flavonols and proanthocyanidins were calculated for some genotypes and also the ratio of flavonoid copigments (flavonols and proanthocyanidins) to anthocyanins (Table 3.2). There was a wide range of copigmentation values with most genotypes being between three and eleven, but in all cases the ratio of copigment to anthocyanin was greater than one. Even when other genotypes were examined (for which proanthocyanidins were not measured) there were none which had less copigment that anthocyanin. The breeding line 4 last had a very high ratio of 160 which was due partly to high flavonol levels but also low anthocyanin concentrations. The genotypes which had a low copigmentation value (e.g. 'Oregon Red') had high anthocyanin concentrations rather than low flavonol or proanthocyanidin concentrations.

Colour measurements

Chromameter measurements were made on the skin of 33 apple genotypes and showed large differences in L^*, a^* and b^* values (Table 3.3). L^* values, or the lightness, ranged from a low of 26.8 in Lawfam actually indicating a dark fruit, through to a high of 69.2 for 'Golden Delicious' which was the lightest fruit. The a^* values (green-red) varied from -17.8 in 'Granny Smith', a very green fruit, up to a high of 37.6 for 4/1 indicating a very red fruit. All the b^* values were positive and ranged from 1.5 in 'Red Cox' through to 49.6 in 'Maling'. When apples are laid out on a CIELAB grid a pattern, where different coloured fruit are positioned on the colour scales, can be observed (Plate 3.2). Fruits of similar colour clustered together, with similar a^* and b^* values. 'Oregon Red' which was the darkest red apple, appearing very dark red almost bluish black, had a fairly low a^* value (11.6 & 9.4) compared to many other red apples. This cultivar also had a low b^* value (3.6 & 2.0) indicating a reasonable blue component in the colour. Those fruit which had the bluish-black tint typical of 'Red Delicious' all had low b^* values ('Lawfam' 3.3 & 4.6, GS 109 3.9, 'Red Cox' 1.5). In many cases an apparent darkening in red colour, or blueing, was marked by a diagonal shift with a move left towards green (lower a^* value) and down towards the blue (lower b^* value). Green and yellow fruit were in a very different location on the grid

Table 3.2: Concentrations of flavonoid groups and copigmentation potential for apple genotypes

Apple genotype	Anthocyanin (mM)	Flavonol (mM)	Proanthocyanidin (mM)	Ratio (F+P)/A[a]
1/1	4.12	19.43	13.69	8.05
1/21	0.69	22.81	4.80	40.04
3/11	1.10	21.34	5.90	24.73
3827	1.64	13.05	5.46	11.30
4926	2.46	17.55	7.42	10.13
4/17	2.25	11.24	6.08	7.71
4 last	0.21	29.46	4.02	160.34
'Granny Smith'	0.00	10.99	6.20	-
GS 150	2.91	17.46	8.66	8.97
'Ikorokavka Alajah'	1.65	12.61	5.68	11.10
J997	4.26	16.29	6.26	5.29
'Lawfam'	3.08	31.27	4.99	11.79
'Oregon Red'	12.96	18.23	19.29	2.89
'Regal Gala'	6.03	12.60	9.16	3.61
S92 Just	4.68	10.01	8.79	4.02
'Splendour'	3.48	15.16	10.97	7.51

[a] F=flavonol, P=proanthocyanidin, A=anthocyanin

compared to red fruit having low or negative a^* values and high b^* values.

To provide an accurate description of colour manipulation of the L^*, a^* and b^* values is required with calculation of the hue (or hue angle) and chroma. In most cases the a^*/b^* values were negative for green fruit, close to zero for yellow fruit and positive for red fruit increasing with the intensity of red. There were a few exceptions; for example for 'Granny Smith' the a^*/b^* value was only -0.53 although because it was so green a lower value would have been expected. Conversely *Malus sylvestris* had a ratio of -6.8 although it was yellow and should have had a value close to zero. The reason for these odd values appears to be high b^* values in both cases.

The hue angle was calculated for all the genotypes and can be converted into ISSCC-NBS colour names (Figure 3.4). The majority of the genotypes (74%) have a hue angle between 0 and 45° with most being 20-30° (red) and some closer to 45° being classified as orange red. The lowest hue angles were for 'Red Cox' and 'Oregon Red' at 10.76° and 11.86° respectively which classes them as purplish red. A small group of genotypes (12%) had hue angles between 45 and 90° and were orange to yellow. 'Fuji'

Table 3.3: CIE $L^*a^*b^*$ values and the calculated colour attributes of the skin of apple genotypes and species measured with a Minolta chromameter

Apple genotype or species	L^* (Value)	a^*	b^*	a^*/b^*	Chroma ($\sqrt{a^{*2}+b^{*2}}$)	Hue (b^*/a^*)	Hue Angle ($\tan^{-1} b^*/a^*$)
1/1	39.9	24.0	10.4	2.31	26.16	0.43	23.27
1/21	43.4	31.9	17.0	1.88	36.15	0.53	27.92
3/11	48.8	27.3	14.8	1.84	31.05	0.54	28.37
3/16	48.0	28.5	21.7	1.31	35.82	0.76	37.23
4926	49.9	12.1	16.5	0.73	20.46	1.36	53.67
4/1	35.3	37.6	21.7	1.73	43.41	0.58	30.11
4/7	44.8	26.7	15.1	1.77	30.67	0.57	29.68
4/17	45.9	20.9	16.4	1.27	26.57	0.78	37.95
4 last	47.0	21.1	19.2	1.10	28.53	0.91	42.30
666	52.8	23.3	24.4	0.95	33.74	1.05	46.38
'Fuji'	46.4	12.6	14.8	0.85	19.44	1.17	49.48
'Golden Delicious'	69.2	-13.5	34.9	-0.39	37.42	-2.59	111.11
'Granny Smith'	60.7	-17.8	33.3	-0.53	37.76	-1.87	118.14
GS 109	37.7	13.5	3.9	3.46	14.05	0.29	16.17
GS 150[1]	40.7	33.9	16.4	2.07	37.66	0.48	25.64
GS 150[2]	41.9	31.1	16.7	1.86	35.30	0.54	28.37
Hyde 5/22	37.6	22.4	10.4	2.15	24.90	0.46	24.70

Table 3.3: *continued*

Apple genotype or species	L^* (Value)	a^*	b^*	a^*/b^*	Chroma ($\sqrt{a^{*2}+b^{*2}}$)	Hue (b^*/a^*)	Hue Angle ($\tan^{-1} b^*/a^*$)
Hyde 5/30	42.8	24.8	12.0	2.07	27.55	0.48	25.64
J997[1]	39.2	25.0	11.2	2.23	27.39	0.45	24.23
J997[2]	36.8	31.6	14.2	2.23	34.64	0.45	24.23
'Lawfam'[1]	26.8	14.7	4.6	3.20	15.40	0.31	17.22
'Lawfam'[2]	33.8	12.5	3.3	3.79	12.93	0.26	14.57
'McKenzie'	57.9	4.9	23.9	0.21	24.40	4.88	78.42
'Maling'	64.4	-12.7	49.6	-0.26	51.20	-3.91	104.35
Malus sylvestris	65.2	-3.0	44.1	-6.80	44.20	-14.7	93.98
'Oregon Red'[1]	37.3	11.6	3.6	3.22	12.15	0.31	17.22
'Oregon Red'[2]	35.0	9.4	2.0	4.70	9.61	0.21	11.86
'Red Cox'	36.3	7.9	1.5	5.27	8.04	0.19	10.76
'Red Delicious'	28.8	19.3	6.6	2.92	20.40	0.34	18.78
'Regal Gala'[1]	38.9	20.9	8.9	2.35	22.72	0.43	23.27
'Regal Gala'[2]	39.7	25.3	11.5	2.20	27.79	0.45	24.23
'Seedling Gavin'	31.6	11.7	4.3	2.72	12.47	0.37	20.30
'Splendour'	47.7	21.7	13.1	1.66	25.35	0.6	30.96
'Sturmer'	60.3	-17.2	20.6	-0.83	26.84	-1.20	129.81
LSD 5%	3.8	5.7	3.5				

Plate 3.2: Apple genotypes placed on a $L^*a^*b^*$ grid

1 'Golden Delicious', 'Maling'

2 'McKenzie'

3 GS 150, J997

4 'Regal Gala'

5 'Red Cox', 'Oregon Red', 'Lawfam'


1
2
3
4
5
35

was in this category although visually it was described as bronze (mixture of red and green). The final group (15%), including 'Golden Delicious' and 'Granny Smith', had hue angles of between 90 and 130° being greenish yellow. As expected there were no genotypes with hue angles greater than this which would put them in the blue region.

According to the guidebook for the Minolta chromameter when the colour aspects of chroma, hue and value are combined the colour of the fruit can be expressed in general terminology using colour obtained from the hue angle (Figure 3.4) and a modifier calculated from value and chroma (Figure 3.5). Thus 'Lawfam' ($L^* = 26.8$, $a^* = 14.7$, $b^* = 4.6$) would be described as being dark red purple and 'Golden Delicious' ($L^* = 69.2$, $a^* = -13.5$, $b^* = 34.9$) brightish yellow. The descriptions obtained in this way generally match well the descriptions obtained by visual means (as given in Appendix A).

Correlation of $L^*a^*b^*$ with pigment content

Once that the two types of colour measurements had been obtained, Minolta $L^*a^*b^*$ values and quantitative estimations of the individual pigments, these two sets of data could be correlated to try to determine if there was any relationship between them (Table 3.4). Anthocyanin content was correlated initially with the a^* values, since this was a measure of red colour, but there was no significant correlation (R^2=0.02) between the two sets of values for the different genotypes and species. Some genotypes such as 'Oregon Red' had very high anthocyanin content (3.25 & 4.34 mg g^{-1} FW) compared to Seedling Gavin (1.18 mg g^{-1} FW) but the two had similar a^* values. Other genotypes that had similar anthocyanin content, such as GS 109 (1.34 mg g^{-1} FW) and J997 (1.44 mg g^{-1} FW), had very different a^* values (13.5 and 31.6 respectively). A number of different models derived from the various chromaticity values were applied all of which showed a greater correlation with anthocyanin content than the a^* value alone (Table 3.4)

Chlorophyll concentration was correlated with a^*, since it is partly a measure of the green component, and a value of R^2=0.35 was obtained. A better correlation might have been expected between carotenoid content and the b^* values since b^* is a measure of the yellow-blue colour and since there is no blue pigment present it may simply have given a measure of yellow aspect. However the R^2 value was 0.04 which suggests that there was a significant blue component influencing the colour.

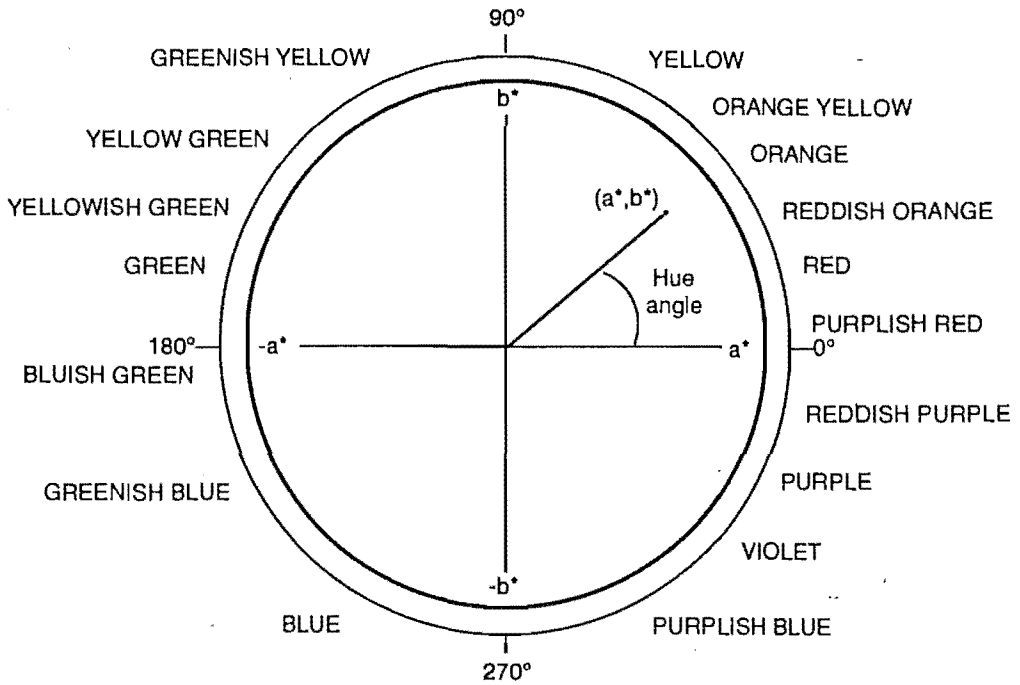


Figure 3.4: Hue sequence and hue angle orientation on a CIELAB diagram with ISCC-NBS colour names (from Voss, 1992)

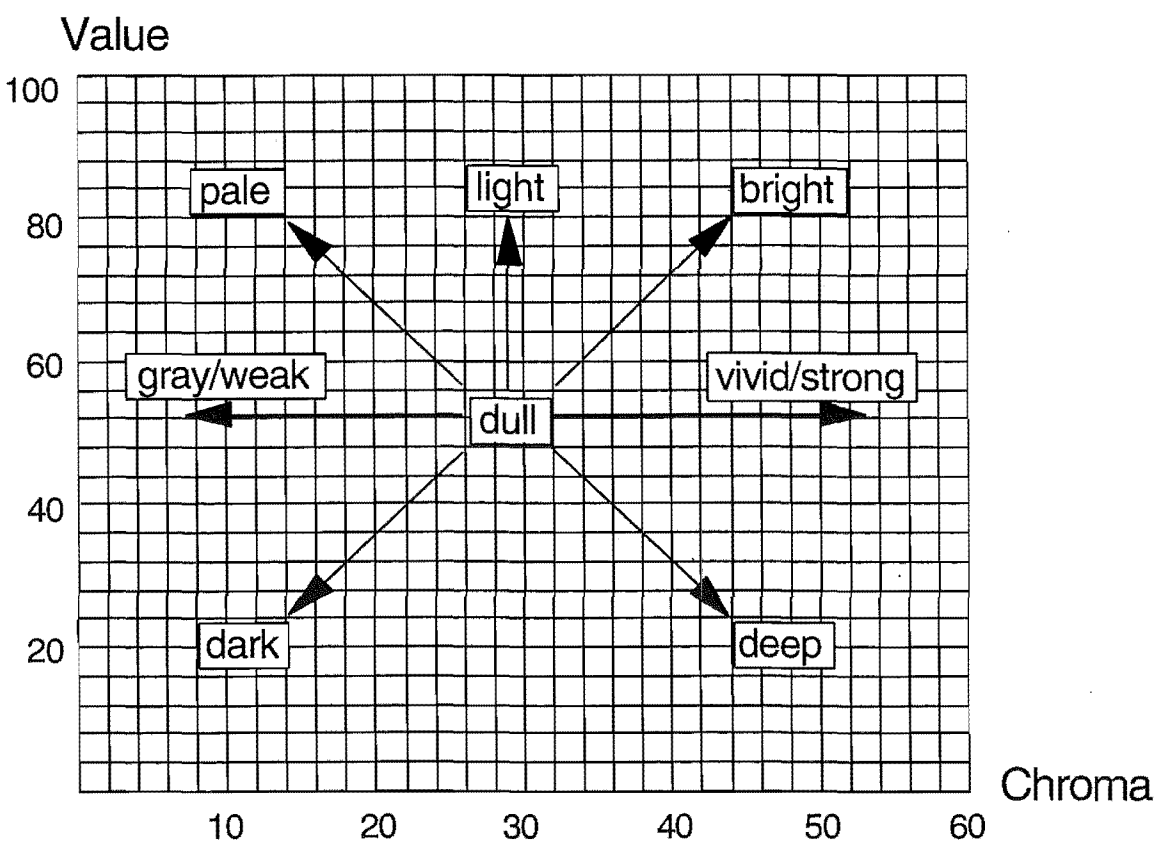


Figure 3.5: Chart of modifiers related to colour tones (value and chroma)

Table 3.4: Coefficient of determination (R^2) for selected regression models relating chromaticity values to anthocyanin content in apple genotypes and species

Variable	R^2
Simple linear models	
L^*	0.41
a^*	0.02
b^*	0.45
hue angle	0.35
chroma	0.41
a^*/b^*	0.64
$(a^*/b^*)^2$	0.64
$L(a^*/b^*)^2$	0.62
$a^*(a^*/b^*)^2$	0.55
More complex models	
L^*,a^*,b^*	0.55
$(a^*/b^*)^2$, $L(a^*/b^*)^2$	0.64
$L^*,(a^*/b^*)^2$	0.67
L^*,hue,chroma	0.51

3.4 Discussion

Pigment levels and colour interaction

All the apple genotypes and species examined here showed the same profile of flavonoids with the main variation being in the total levels of each of the groups (anthocyanins, flavonols and proanthocyanidins). Thus, flavonoid composition is not an explanation for variation in shade of red colour. There were large variations in the chlorophyll and carotenoid concentrations in the different genotypes and species and these plastid pigments are well known to have a modifying effect on flower and fruit colour (Peach, 1955; Gross, 1987; Teynor *et al.*, 1989a). Since the plastid pigments varied independently of each other and of the flavonoids the modifying effect that they had on colour would vary considerably between genotypes. In those genotypes that had low anthocyanin concentrations the presence of chlorophyll gave a bronze appearance as in 'Fuji' and 'Sturmer'. However, where anthocyanins were higher it was difficult to determine the effect of the chlorophyll since the fruit did not appear bronze but the presence of the chlorophyll probably darkened the actual appearance. Where carotenoids were present together with anthocyanins, but in the absence of chlorophyll, the fruit tended to appear orange-red. This type of effect has been observed in orange flowers of chrysanthemums (Teynor *et al.*, 1989a). Thus the combination of these three groups of pigments, anthocyanins, carotenoids and chlorophyll, probably results in some of the variation in colour hues seen in apple skins. However, this does not explain all the variation particularly the blueing observed in some genotypes.

Copigmentation

The copigmentation ratios of all the apple genotypes examined were greater than one. Since copigmentation effects have been shown to occur with equimolar or higher copigment/anthocyanin ratios (Asen *et al.*, 1972) it is likely that copigmentation was occurring in all apple genotypes and species. Further investigation of copigmentation in apple skin has been carried out (Lancaster *et al.*, 1994). The λ_{max} was determined for a range of apples exhibiting variation in the shade of red to determine how much they differed from the standard of cyanidin-3-galactoside. It was found that the λ_{max} did not differ significantly between genotypes and there was no trend of increasing λ_{max} with darker red skin. However the mean λ_{max} for apple cells was 550 nm which is higher than the λ_{max} of 523 nm for cyanidin-3-galactoside in aqueous solution at pH 3.0. Thus, it would appear that copigmentation occurs in all genotypes resulting in the higher λ_{max} but does not influence colour variation between genotypes. Jurd and Asen (1966) reported that simple mixing of cyanidin-3-glucoside and quercitrin did not lead to copigmentation but addition of aluminium salts did. From this it could be postulated that the level of Al^{3+} present could influence copigmentation. If this was so then apple colour would vary depending on the location but the same apple cultivar grown at different locations show the same shade of red although some variation is often observed in the intensity. Thus copigmentation, together with the interaction with

Al^{3+}, cannot be an explanation for the variation in red hue seen in apple cultivars, unless the level of Al^{3+} in the fruit skin was under genetic control.

Asen *et al.* (1972) observed that for cyanidin at pH 3.16 an increase in the overall pigment concentration from 10^{-4} to 10^{-2} M produced a 300-fold increase of the absorbance at the absorption maximum in the visible spectrum and this was attributed to self-association of the pigment. However the importance of self-association in *in vivo* expression of fruit colour has not been determined and there may also be competition between self-association and copigmentation (Macheix *et al.*, 1990). It may have a significant effect in cultivars such as 'Oregon Red' where the copigment/anthocyanin ratio is not exceptionally high but the anthocyanin concentration was 10^{-2} M which is around the critical concentration that has been postulated where the anthocyanins are nearly planar and form molecular aggregates (Harborne, 1988).

Other factors influencing colour

The spectral absorbance of anthocyanins is also influenced by pH. The pH of apple epidermal tissue has been determined to be between 3.6 and 4.0 but there are no published measurements on the pH of skin of different genotypes. The relative uniformity of λ_{max} for the different genotypes argues against pH as an explanation for the different colours of red in different genotypes. This is supported by data from extraction of the apple anthocyanins at varying pH. Over the pH range 1 to 7 the apple extracts remained the same red colour although there was some fading of colour at pH greater than 4. This would suggest that there was some stabilizing mechanism, as normally above pH 3 a change in the shade of red or fading of the colour (anthocyanin) would be expected.

It is unlikely that effects of metal chelation are a major influence on apple colour. As discussed above this would vary with different soil types and would mean variation in colour with location rather than the variation between cultivars which is observed. Some variation is observed within a cultivar depending on location and season but there is still a typical colour for a particular cultivar. The variation observed is generally only in intensity of colour rather than differences in hue. Thus, it would appear that it is genetic factor(s) rather than environmental influence(s) which controls the shade of red colour. However, the intensity of red colour is controlled by both genetic and environmental influences.

Control of fruit colour

Three general classes of genes have been suggested by Harborne (1967) to be involved in flavonoid biosynthesis, these control chemical structure, general production and distribution of the pigments. A fourth general class was suggested by Stewart *et al.* (1969) which were genes that affect colour through controlling the physical and/or chemical state of the pigment in the living cell. For flowers this class of genes was apparently as important as the other three in determining colour. Included in this fourth

category are genes affecting pH, metal chelation and copigmentation, factors known to change the colour of individual anthocyanins. For apples the genes controlling the chemical structure are not an influence on colour since there are no variations in structure of compounds observed in different genotypes. The distribution of flavonoids within the apple is fairly constant, with the exception of species such as *Malus pumila niedzwetzkyana* which has pink flesh, and most of the rest of the plant is also coloured.

The class of genes that involves the state of the pigment in the tissue, although important, does not appear to be responsible for the significant differences in colour. Copigmentation has been shown not to be the factor responsible for the range of colours seen in different apple fruit. The class of genes that appear to be of the most importance are those involved in biosynthesis, that is those affecting the levels of flavonoids in the skin. These include the genes for the enzymes involved in the biosynthetic pathway and those involved with the control of production such as the UV light thresholds. It has been shown that the minimum energy requirement for pigment synthesis varied considerably with cultivar (Proctor, 1974). Thus, the intensity of red colour of a particular apple cultivar appears to be a function of severally genetically controlled factors which are the energy requirement for pigment synthesis, vacuolar size and pigment distribution. Environmental influences will also affect this and a cultivar may not reach its full pigmentation potential if conditions are not ideal.

Correlation of $L^*a^*b^*$ with pigment content

Singha *et al.* (1991a) studied the relationship between anthocyanin concentration and chromaticity values and found little correlation between anthocyanin levels and a^* (R^2=0.10). Although this is a slightly better correlation than that obtained here it is still not significant. It is not really surprising that with apples there is not good correlation between a^* values and anthocyanin content because, by definition, the a^* values represent the colour component on the green-red axis and even a minor contribution of a green element could considerably reduce the overall measured a^* values. Most apples have both a green component (chlorophyll) and a red component (anthocyanin) in their colour and these two vary independently. Thus, a dark red apple, such as 'Red Cox', will not give as high an a^* value as 'Royal Gala' despite having similar anthocyanin contents and this is because 'Red Cox' has a much higher chlorophyll content which "pulls it back" on the green-red axis.

In some fruit such as tomato [*Lycopersicon esculentum* var. *cerasiforme* (Duval) A. Gray] and cranberry (*Vaccinum macrocarpon* Ait.) there is good agreement between visual scores, red pigment content and Hunter colour values (R=0.997) (Francis & Clydesdale, 1970; Larrigaudiere *et al.*, 1991). For tomato the a^* value is sufficient to characterize maturity stages. However, in each of these fruits the pigment composition is simpler and major changes occur in only one pigment, whereas in apple skin there are three pigments accumulating independently, namely chlorophyll, carotenoids and anthocyanins.

For ripening peaches [*Prunus persica* (L.) Batsch] major changes in colour were reflected by no change in b^* but an increase in a^* (Delwiche and Baumgardner, 1985; Byrne *et al.*, 1991). However, the

increased a^* value was brought about by changes in two pigments; a decrease in chlorophyll and an increase in anthocyanin. Similarly for watermelon [*Citrullus lanatus* (Thunb. Matsu. & Nakai] fruits an increase in a^* values during ripening was brought about by a loss of chlorophyll. Carotenoid levels, and thus the b^* values, were constant but the fruit appeared orange due to the unmasking of the carotenoids as the chlorophyll disappeared (Corey & Schlimme, 1988). Genotypes such as 'Oregon Red Delicious', which have high anthocyanin levels, and thus would be expected to give a higher a^* value also have high chlorophyll levels, which reduce the a^* value.

The modifying effect of chlorophyll on anthocyanins has also been monitored for eggplant (*Solanum melongena* L.) where the darkest purple fruit contained high levels of chlorophyll and anthocyanins (Nothmann *et al.*, 1976). Likewise, in petals from tulip (*Tulipa gesnerana* L.) and chrysanthemum [*Dendranthena grandiflorum* (Ramat.) Kitamura], carotenoids modify the appearance to red-orange when present together with anthocyanins (Nieuwhof *et al.*, 1989; Teynor *et al.*, 1989a & b).

Singha *et al.* (1991a) tested a number of models to attempt to relate chromaticity values to anthocyanin content which gave poor to satisfactory results. Even though the range of genotypes examined here was much more diverse the correlations obtained with these same models are very similar. The best correlation was between anthocyanin and $(a^*/b^*)^2$ where $R^2 = 0.64$ and a more complex model L^*, $(a^*/b^*)^2$ where $R^2 = 0.67$. These relations were derived empirically and have no theoretical basis. Similar models applied to data collected in this study gave similar values. Thus, no real comparison could be made between anthocyanin concentration and colour measurements.

No correlation was observed between chlorophyll content and a^* values or carotenoids and b^* values. It was not really surprising that there was not a good correlation with chlorophyll content (green) since the red factor was not constant and this also had an influence on the a^* values. A higher correlation might have been expected between carotenoid concentration and the b^* value due to absence of a specific blue pigment in apples. This lack of correlation is significant in that it shows that there is a strong blue colour component in some genotypes, but it is not a constant factor. This blueing could arise from self-association or copigmentation effects. These appeared to occur in genotypes, such as 'Oregon Red' and 'Regal Gala', where anthocyanin concentrations were high.

Various other problems arise during the comparison of the two methods of colour estimation and one of these relates to the area of colour estimated. With the Minolta Chromameter the area of colour measured is very small (8 mm diameter) whereas a much larger area of tissue is required for analysis. Thus, in order to make direct comparisons, a much large number of readings need to be taken with the chromameter since variation within the apple can be quite high. Another problem is that during quantitative analysis the tissue is disrupted and the physiochemical environment of the pigments is altered; this may affect the colour by changing copigmentation and self-association effects of the anthocyanins.

Attempts to relate chromaticity values to pigment composition were unsuccessful because, unlike some fruit, apple colour was not due to a single group of pigments but three groups, each of a different colour, present in most genotypes. Carotenoids (yellow), chlorophyll (green) and flavonoids (red) are

present in apple skins and each vary independently to dictate the final colour of the fruit. Where carotenoids were present in high levels, but chlorophyll was absent, the red colour was modified making it appear more orange such as in 'Gala' genotypes. High chlorophyll levels tended to darken the red colour or make the apple appear bronzy, depending on the level of anthocyanin. In the absence of these plastid pigments, such as in 'Ikorokavka Alajah', the fruit had a pinkish appearance.

Special reflectance spectrophotometers have been used to measure green (546 nm) and red (640 nm) reflectance of a rotating object and this has been especially suitable for measuring changes in developing fruits in which green and red are dominant (Gross, 1987). Measurements of apple colour were made using a Chromatic Colour Image Analysis system which gave red, blue and green colour measurements. However, detection was not sensitive enough in the green and blue regions and red values showed no correlation with anthocyanin content.

Thus, it is difficult to obtain objective measurements of colour in apples which correlate with anthocyanin concentration in different genotypes and species. Visual comparisons of fruit colour are still the most useful when a range of apple genotypes, which vary in shades of red, are to be examined. Objective measurements of colour still have their uses and may be used as an indicator of maturity for a single cultivar. Although in a single cultivar there are still changes in all three pigment groups the pattern of change within a cultivar is consistent whereas the developmental changes are different between cultivars. This is investigated in Section Four.

3.5 Conclusions

In summary, what are the key factors influencing colour? The composition of the flavonoid pigments, and in particular the anthocyanins, did not differ significantly between genotypes. The only differences in flavonoids were quantitative, not qualitative, resulting in the variation in intensity or red colour but not difference in hue. Since there were high levels of flavonols and proanthocyanidins in all apple genotypes and species copigmentation probably occurred but was a constant factor. It has a role in stabilizing anthocyanin colouration but it probably does not influence colour variation. Where anthocyanin levels were high self-copigmentation may be occurring resulting in the bluish red colour that is seen in cultivars such as 'Oregon Red'. The differences in hue are more likely to be due to the visual blending of chlorophyll, carotenoids and anthocyanins.

The level of anthocyanin production is the major influence on the intensity of red colouration. All apples accumulate flavonoids up to and including the anthocyanins. Even non-red genotypes and species have the ability to produce anthocyanin under extreme conditions so they do not lack the gene(s) for its production. Therefore, the key step(s) is probably the genetic control of the final stage of biosynthesis (conversion of leucocyanidin to cyanidin) that dictates the level of anthocyanin produced. However the genes controlling total flavonoid production are probably also important in influencing colour by controlling the total amount of pigment that can be synthesized by the flux through the whole pathway. The intensity of the red colour is a function of the energy requirement for pigment synthesis, vacuolar size, pigment distribution and also the influence of environmental factors which dictate whether full pigmentation potential can be reached.

Section 4 - DEVELOPMENTAL CHANGES IN PIGMENT COMPOSITION AND LEVELS

4.1 Introduction

4.1.1 Developmental changes in pigments

Although the final levels of pigments reached will have an influence on fruit colour at maturity, it is important to determine how these levels are reached. The changes in fruit colour observed during development and ripening may be the result of a number of different changes taking place in the composition and concentration of the pigments. These changes may be due to: (1) accumulation of a class(es) of pigments during growth and maturation; (2) increased or new synthesis of a class of pigments during the ripening process; (3) fading or disappearance of a particular colour which may result from the dilution of a pigment as the cells expand or degradation of existing pigments which may be either chemical, enzymic or photodegradation; (4) a change in the physiochemical environment of the pigments resulting in a colour change although no quantitative change occurs. This may be a change in vacuolar pH or the presence of other compounds, such as metal ions, or any of the other factors discussed in Section 3.1.3 that may have an influence on the colour of the anthocyanins.

Systematic study of the pigment changes during fruit ripening began only recently, although seasonal colour changes in apple were described a long time ago (Haller & Magness, 1944). It was gradually realized that colour and quality were not casually related and fruit colour became to be considered as a quality index and research on the subject intensified. For commercial purposes the fruit colour rating, by physical methods, has been intensively used to put fruit into categories of ripeness. Red colour was often used but was not always a good indicator of maturity as it can vary markedly between fruit depending on tree position, different orchards and seasons. Thus background colour (yellow and/or green) is often used as an indicator since it is less variable (Kingston, 1991). For a deeper understanding of the colour changes during the ripening process, more research is necessary.

4.1.1.1 The flavonoids

Accumulation of phenolic compounds varies strongly in relation to the physiological state of the fruit and is a result of the equilibrium between biosynthesis and further metabolism including turnover and catabolism (Macheix *et al.*, 1990). Important control mechanisms in phenolic metabolism are the control of the amount and regulation of enzyme activity (which is examined in more detail in Section Five), compartmentation of the enzymes, availability of precursors and intermediates, and integration in the differentiation and developmental programs in the producer organism (Luckner, 1980). Most of the work

done on the regulation of the flavonoid biosynthetic pathway has been on the role of light in regulation. Little work has been undertaken on the developmental regulation and most has focused on the regulation during ripening (Lancaster, 1992). There are still major gaps in the understanding of these developmental programs although considerable progress has been made in the last 25 years.

During maturation of most fruits the anthocyanins are synthesized at an increasing rate, especially near maturity, reaching a maximum in the fully ripe fruit (Gross, 1987). These changes have been investigated for many fruit including the apple however, changes in the other flavonoids that often accompany them are not so well documented. In some cases it has been noted that the concentration of phenolics decreases as the fruit matures but the amount per fruit increases (Craft, 1961; van Buren, 1970) but the specific changes in individual compounds or classes of compounds have rarely been studied. Recording the qualitative and quantitative changes in the flavonoids during maturation serves a number of purposes. Total anthocyanin content is often considered to be an index of maturity, and is also an important quality parameter (Gross, 1987). In many fruit the anthocyanin concentration of the skin increases during development, particularly in the later stages of ripening and following these changes may lead to a deeper insight into the biosynthetic mechanism involved (Gross, 1987). In studies of anthocyanins in flowers it has been found that during development the sequences are characterized by a progression from lesser to greater complexity, both in anthocyanidins and glycosylation pattern (Harborne, 1967). These types of changes have not been as well documented for fruit.

Anthocyanins

There have been various reports on developmental changes in flavonoids in apples but most research has been centred on total anthocyanin changes during ripening (Creasy, 1968b; Chalmers *et al.*, 1973; Faragher & Brohier, 1984). There are actually two peaks of anthocyanin formation in apples: (1) a first peak during the phase of intense cell division in the fruit, which has been neglected so far because it is economically unimportant and of unknown biological significance; (2) a second peak coinciding with the ripening of red cultivars (Saure, 1990). Both types of anthocyanin formation occur under a variety of environmental conditions pointing to endogenous control (Saure, 1990).

As a rule young fruitlets exhibit intense temporary bronze-red colouration even in cultivars, such as 'Granny Smith' and 'Golden Delicious', not inclined to anthocyanin formation during maturation. It has not yet been determined whether the anthocyanins during early fruit development are identical to those in ripe fruits but as apples grow the reddening disappears and whether this is due to breakdown, dilution or a change in the state of the pigment is unknown. It has been suggested by Bömeke (1959) that the intense colouration of young fruit is due to the cooler nights and the fading in summer is a consequence of the warmer weather. However, this still occurs in the tropics where temperatures are high and thus, it is more likely that the anthocyanin formation in young fruit is fully endogenous (Saure, 1990). Young fruit contain high levels of catechins (Mosel & Herrmann, 1974a; Burda *et al.*, 1990) but there are no published reports on the levels and nature of the other flavonoids in the skin of very young apples. High

levels of flavonoids may act as UV protectants to filter out UV light during cell division and prevent nuclear aberration (Lancaster, 1992).

The second peak of anthocyanin synthesis, coinciding with ripening, has been reasonably well reported for a number of apple varieties but has been limited mainly to measurement of total anthocyanin concentration and not composition. There is a steady increase in the anthocyanin content of apple skin observed from verasion onwards (Creasy, 1968b; Chalmers *et al.*, 1973; Faragher & Brohier, 1984) and these changes are also observed in grape, cherry, strawberry, plum and many other red fruit (Melin *et al.*, 1977; Hrazdina *et al.*, 1984; Macheix *et al.*, 1990; Cheng & Breen, 1991). In 'Jonathan' apples anthocyanin content was at a low and constant level for most of the growth of the fruit but this was followed by a three-fold increase over the last month before maturity (Faragher & Brohier, 1984). The same change was reported in 'Cox's Orange' fruit (Knee, 1972) and for 'McIntosh' the increase was slightly larger, being four to five-fold (Creasy, 1968b).

Flavonols

There have been few studies devoted to the variation in flavonols and their glycosides during fruit ripening and this is particularly true for apples. Much of the early work was carried out on non-red varieties such as 'Golden Delicious'. Quercetin glycoside concentration remained relatively constant during the maturation of 'Golden Delicious' apples (Workman, 1963; Gorski & Creasy, 1977) and then increased during storage (Gorski & Creasy, 1977). Workman (1963) calculated the total amount of quercetin glycosides per apple and found that it increased during maturation because of the growth of the fruit however, only total quercetin levels were measured and changes in individual glycosides were not reported. Dick (1986) noted that the flavonol content dropped during maturation of a number of apple cultivars. Individual quercetin glycosides varied but the total concentration remained relatively constant until the onset of the climacteric when levels decreased significantly while fruit were still on the tree. Fruits which were picked and kept in cold storage for two months showed a strong increase in quercetin glycosides. Phloridzin changed in a similar way in the same fruits.

Proanthocyanidins

Changes in specific proanthocyanidins have been studied for several varieties. Burda *et al.* (1990) studied 'Rhode Island Greening' apples with relation to enzymic browning during development and found that (-)-epicatechin was highest in the skin of young fruit and decreased to remain constant during maturation and storage. Levels of procyanidin B2, quercetin and phloretin glycosides and chlorogenic acid were also recorded. Developmental changes in the flavan-3-ols were reported by Mosel & Herrmann (1974a) for 'Schöner von Boskoop' and 'Golden Delicious' apple cultivars, who found that (-)-epicatechin and (+)-catechin concentrations rose sharply until an age of 2 to 3 months and then declined. It was noted that (+)-Gallocatechin was also sometimes formed in the last stage of maturity or ripening. In another report catechins were high in young fruit peaking at 40 days after flowering and then decreasing rapidly

stabilising at a very low level until maturity with (+)-catechin and (-)-epicatechin varying in a similar manner (Macheix *et al.*, 1990).

The flavonoids, and in particular the anthocyanins, are important in colour and in this project both the qualitative and quantitative changes were monitored during the development of the apple fruit. There is no published work on the concentrations of all the major flavonoids in apples during development despite their close biosynthetic relationship. Most of the existing work, other than on the anthocyanins, has focused on green or yellow fruit and not on red cultivars. In this study the changes in concentration and total amount of the individual anthocyanins, flavonols (quercetin glycosides) and proanthocyanidins were determined in the skin of yellow, green and red local cultivars. Comparison of all the flavonoid changes occurring in the skin of non-red versus red apple cultivars may be informative in elucidating all the steps involved in anthocyanidin biosynthesis and hence reddening.

There are three main ways of expressing the concentration of flavonoid compounds in fruits: (1) on the basis of fresh weight; (2) dry weight basis; and (3) amount per fruit. Expression on the basis of fresh weight is the most commonly used. This gives a fairly good representation of the vacuolar concentration of the flavonoids since these compounds are located there and a large percentage of the water is located in the vacuole. Expression in terms of dry weight can be useful to eliminate variations in water between two samples but there were no significant differences between apple skin samples and so this was not necessary. This type of measurement can be artificial since a large proportion of this material is accumulated in compartments which do not contain flavonoids. Calculation of the amounts of flavonoids per fruit is often useful to accompany the other two methods of expression. For a fruit like apples this reflects a measurement of the surface area of the fruit since the flavonoids are located almost entirely in the skin. For the purpose of this study flavonoids were expressed in terms of fresh weight and total amounts per fruit.

Flavonoid degradation and turnover

In addition to synthesis of the flavonoids another factor which has an effect on final levels is degradation, which may be chemical or enzymic but only limited data is available on the degradation and turnover of the flavonoids particularly in fruit. The turnover and catabolism of the flavonoids have been reviewed by Barz and Hösel (1975), Barz and Köster (1981) and Barz *et al.* (1985).

In other plants it appears that turnover rates of the anthocyanins are very variable. No evidence of degradation was found in *Matthiola incana* buds (Dangelmayr *et al.*, 1983). Turnover of 3-6% per day during intense anthocyanin accumulation was observed in *Sinapis alba*, however when synthesis was low the rate of synthesis and degradation were very similar resulting in a constant anthocyanin level (Zenner & Bopp, 1987). *Petunia hybrida* showed a high turnover rate of 45% per day (Stafford, 1990). Destruction by peroxidase of various flavonoids, including flavonols, has been reported (Barz *et al.*, 1985). There is little evidence to suggest that proanthocyanidin oligomers are degraded but dimers and flavan-3-

ols may be different (Stafford, 1990) and may be oxidized by plant polyphenoloxidases to amorphous brown pigments. Phloridzin has also been reported to be degraded by hydroxylation to 3-hydroxyphloridzin followed by oxidation to a stable dimeric quinone (Goodenough *et al.*, 1983).

Many fruit possess an enzyme system capable of decolourising anthocyanins (van Buren *et al.*, 1960). An enzyme system capable of anthocyanin degradation has been detected by Schmid (1967); this first removed the sugar followed by oxidative ring opening of the aglycone. Activity of degrading enzymes was much less in highly coloured 'Jonathan' apples as compared with 'Golden Delicious' and 'Cox's Orange Pippin'. Activity increased on storage and it has been suggested that the enzyme was of microbial origin (van Buren, 1970). Chalmers *et al.* (1973) suggested that in immature fruit there is a shortage of galactose and thus there is rapid degradation of the anthocyanidin because it cannot be glycosylated to make it more stable. An increase in galactose prior to harvest allows glycosylation to take place and the anthocyanin transported to the vacuole without prior destruction. Schmid (1967) observed that galactose and lactose, but not glucose, inhibited the enzymatic degradation of anthocyanin in apple peel. However, it has not been demonstrated whether this actually occurs *in vivo*.

Calculations of total flavonoids and the flavonoid groups (anthocyanins, flavonols and proanthocyanidins) per apple were made to provide some information on whether any decreases in concentration were due to dilution of the pigments as the skin expands, or actual degradation was taking place. This does not give information on the actual turnover, the balance between synthesis and degradation but studies on the biosynthesis of the flavonoids in the next section may shed more light on this question.

Influence of external factors

External factors can also influence anthocyanin formation and these have to interact with the endogenous control and may even affect it (Saure, 1990). The effect that these external factors have on the other flavonoids has not been as well studied. Biosynthesis of the anthocyanins in plant tissues either requires light or is enhanced by it (Mancinelli, 1983). More specifically, in apples it is a requirement for synthesis (Siegelman & Hendricks, 1958) and additional light can stimulate further production (Proctor, 1974). The quality of the light is also important and intense colouration of apples occurs at higher altitudes and also after periods of rainy weather. These increases in anthocyanin accumulation have been attributed to a higher percentage of UV light (Magness, 1928). Workman (1963) noted that green 'Golden Delicious' fruit from inside the tree had much lower flavonoid levels than those fruit from the outside of the tree. This was presumably due to the differences in light levels received by the fruit.

Temperature also has a significant effect on anthocyanin accumulation and decreasing temperature in autumn generally coincides with a phase of intense anthocyanin formation (Saure, 1990). Therefore, low temperatures are considered to promote and high temperatures inhibit anthocyanin formation (Creasy, 1968b; Proctor, 1974; Faragher, 1983). Various nutritional factors and cultural practices also have significant influences on anthocyanin formation (Walter, 1967; Saure, 1990). These various external

factors may also interact to produce a wide variety of responses and it has not been determined whether external or endogenous factors are the major influence on final levels. Since these external factors are so variable from year to year it is possible that different patterns of flavonoid change could be observed in different seasons and it is important therefore to gather more than one seasons data. Workman (1963) reported a large difference in the quercetin glycoside levels, from 'Golden Delicious' fruit, over the two years that were sampled although in both years the concentration remained fairly constant. No reasons for these differences were postulated but it is possible that they were due to environmental influences.

4.1.1.2 Plastid pigments

Chlorophyll

As apples mature they lose their green skin colour as chlorophyll is lost. Until maturity chlorophyll is continuously regenerated but once maturation begins chlorophyll production slöws and as chlorophyll disappears other pigmentation, often yellow, appears (Kingston, 1991). This change in background colour from green to yellow is often used as an indicator of maturity but it varies between cultivars and is influenced by a number of factors.

In an early study of colour changes in two apple cultivars, chlorophyll was assessed globally during fruit development. In both cultivars chlorophyll decreased continually during ripening (Workman, 1963) and since then loss of chlorophyll has been frequently observed during ripening of apples. This has been associated with the respiration climacteric and may be due partly to an observed increase in chlorophyllase activity (Rhodes & Wooltorton, 1967). Mussini *et al.* (1985) studied chlorophyll changes in 'Granny Smith' during ripening and noted that significant chlorophyll degradation occurred. Decreases in chlorophyll levels have also been reported for 'Cox's Orange Pippin' where they fell fourfold during ripening (Knee, 1972). In 'Golden Delicious' chlorophyll concentration also decreased, by approximately threefold, during the two months prior to harvest (Gorski & Creasy, 1977).

Carotenoids

Studies on chlorophyll changes during fruit development have often been accompanied by measurements of the carotenoids. Mussini *et al.* (1985) noted that carotenoids decreased during development in 'Granny Smith' but the drop was not as dramatic as that of chlorophyll. Knee (1972) carried out a comprehensive study of the pigment changes occurring in developing 'Cox's Orange Pippin' apples and found that total carotenoids increased fourfold during ripening. In a study on 'Golden Delicious' by Gross *et al.* (1978) it was found that during early development carotenoid content decreased from 18.2 $\mu g\ g^{-1}$ FW to a minimum of 7.3 $\mu g\ g^{-1}$ FW, then increased and remained nearly constant at 13 $\mu g\ g^{-1}$ FW until ripening. There was a substantial change in the carotenoid pattern. Increases in total carotenoid content have been observed in ripening apples of 'McIntosh' (Francis *et al.*, 1955) and 'Golden Delicious' (Workman, 1963). However Zelles (1967) observed that the carotenoid content of 'Golden

Delicious' and a number of other cultivars remained relatively constant. Gorski & Creasy (1977) determined the minimum amount of carotenoids necessary for yellow colour and found it to be 0.3-0.4 $\mu g\ cm^{-2}$ of skin. They found during the development of 'Golden Delicious' fruit carotenoid levels first dropped and then rose back up to their original levels.

Thus, there has been a wide range of patterns of change observed in carotenoid levels during the development of apple fruit. These differences may reflect cultivar differences but there also seems to be environmental influences, since different patterns have been observed in a single cultivar. Therefore, it is important to see exactly what pattern of change occurs in New Zealand-grown cultivars since it is not possible to draw conclusions from previous data. Since chlorophyll and carotenoids are important to the overall appearance of the fruit the changes occurring during development were measured. The period of study was not limited to the month of ripening, as reported by many researchers, but covered a large portion of the fruit's development, from about six weeks after fruit set through to normal harvest at maturity.

4.1.2 Apple fruit development

When studying the changes occurring in the pigments of the developing fruit it is important to consider changes in cell division and expansion. The early peak of anthocyanin formation is associated with cell division (Saure, 1990) and proanthocyanidins have been reported to be synthesized early on during the cell elongation stage of a cells development (Stafford, 1990). Tetley (1930 & 1931) studied the morphology and cytology of developing 'Bramley's Seedling' apples and established that cell division ceased a few weeks after fruit set and thereafter size was mostly due to cell enlargement. This was confirmed by Bain & Robertson (1951) who found that cell division ceased within four weeks of pollination but cell enlargement continued throughout the life of the fruits on the tree. This basic pattern seems to be true for a range of cultivars (Smith, 1950; Denne, 1960 & 1963; Skenne, 1966) although there is some variation in the duration of cell division. Skenne (1966) found that cell division continued for considerably longer in the epidermis than in the cortex. It stopped when the fruit was about 45 mm in diameter or 65-70 days after blossom.

4.2 Materials and methods

Materials

Plant material

Fruit of the cultivars 'Golden Delicious' (yellow), 'Granny Smith' (green) and 'Splendour' (red) were obtained from the Lincoln University Research Orchard, Lincoln, Christchurch, New Zealand. Samples of six apples per cultivar were collected at seven or eight dates during the season, starting as soon as the apples reached a size large enough to sample to process, through to normal harvest time at maturity. The dates of sampling were December 5, January 5 & 26, February 15, March 2, 16 & 29, and April 12, 1989/90. For the second season the dates of sample collection were December 11 & 27, January 14, February 14, March 4 & 21 and April 10, 1990/91.

Methods

Handling of fruit

At each sampling date the fruit appearance, weight and diameter were recorded. Peel was removed carefully, taking care to scrape away underlying cortex, and weighed. Half the peel from each fruit was used for estimation of chlorophyll and carotenoids and the other half for flavonoid estimation. Care was taken so that each sample was representative of the apple and there was no bias to sun or shade side or top or bottom half of the fruit.

Extraction of flavonols and anthocyanins

Extraction and identification of the flavonoids was as outlined in Section 2.2.

Quantification of flavonols and anthocyanins

Samples for the 1989/90 season were separated prior to HPLC using the column chromatography method and the earlier HPLC method (see Section 2.2 - Separation of anthocyanins and flavonols, HPLC of flavonols and anthocyanins, and HPLC of proanthocyanidins). The 1990/91 ('Granny Smith' and 'Splendour' only) samples were quantified by the later, improved HPLC, method (see Section 2.2 - Comparison of Flavonoid Composition). The losses incurred by the column chromatography method were compensated for.

Extraction of proanthocyanidins

As outlined in Section 2.2. Data was only obtained for the 1990/91 season for 'Granny Smith' and 'Splendour'.

Quantification of proanthocyanidins

As outlined in Section 2.2.

Extraction of chlorophyll and carotenoids

Extraction procedure was the same as that outlined in Section 3.2.

Quantification of chlorophyll content

Estimation of chlorophyll was as given in Section 3.2.

Quantification of total carotenoids

Carotenoid estimation was performed as outlined in Section 3.2.

Statistical analysis

Concentration and total amount of quercetin glycosides, proanthocyanidins and cyanidin glycosides were measured for each fruit. Fruit diameter was used to estimate surface area, assuming a spherical shape, and total amounts of each compound calculated for individual fruit. Standard ANOVA established significant differences among cultivars over time, so subsequently each cultivar was analyzed separately. Concentrations for some minor compounds, and amounts per apple for all compounds, were log transformed before analysis to stabilize variances over the season. The angular transformation was applied to the relative amounts (percentages) of the individual components of quercetin glycosides and proanthocyanidins. Graphs were drawn using the same scale used for all analyses so that the LSD's used are those appropriate for comparing means at two dates for a given component. For Figure 4.3(c) and Figure 4.4(c) where concentrations of cyanidin glycosides were near to zero before March each year this data was omitted for computation of LSD's.

The same calculations were made for total carotenoids and chlorophyll.

4.3 Results

Identification of flavonoids

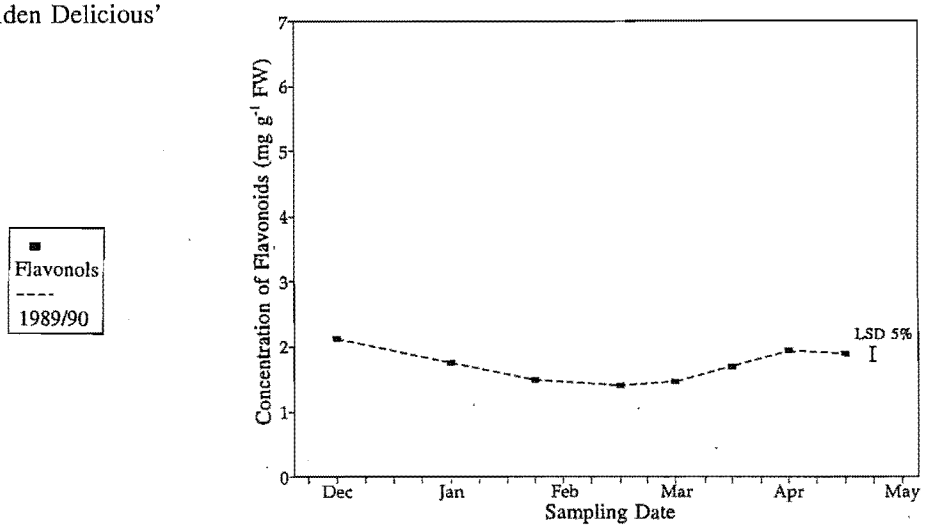
As was observed in Section Two the composition of the flavonoids for all three cultivars was very similar. The flavonols and proanthocyanidins present were the same and were present in similar proportions. The only significant difference was in the levels of anthocyanins with 'Splendour' containing high levels at maturity compared to 'Granny Smith' and 'Golden Delicious' which only contained traces of anthocyanins in very immature fruit. Traces were also present in fruits under high stress conditions such as those at the top outside of the tree exposed to high light levels.

Levels of flavonoids in developing fruit

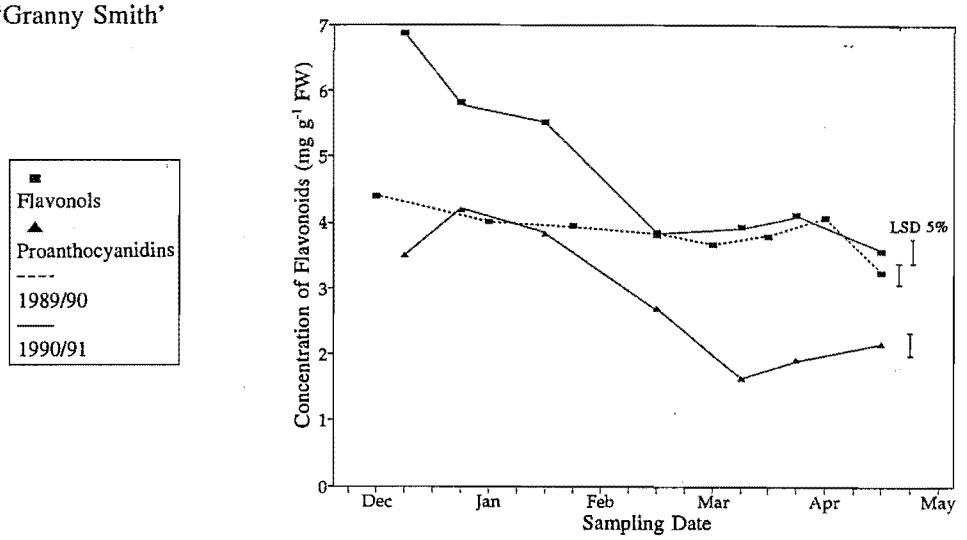
The total flavonoid levels in the three cultivars varied from a low of 1.5 mg g^{-1} FW (excluding proanthocyanidins) in 'Golden Delicious' up to 10.5 mg g^{-1} FW in 'Splendour' which equates to about 4% of dry weight. Quercetin glycosides were the major flavonoids in all three apple cultivars. Concentrations varied with 'Golden Delicious' containing the lowest levels at 1.2-2.1 mg g^{-1} FW, 'Splendour' 2.5-5.5 mg g^{-1} FW, and 'Granny Smith' being the highest at 3.5-7.0 mg g^{-1} FW (Figure 4.1). Proanthocyanidins were present at a concentration of 1.5-4.5 mg g^{-1} FW for 'Granny Smith' and 2-4.5 mg g^{-1} FW for 'Splendour'. Cyanidin glycosides were present in 'Splendour' at a concentration of 0-1.3 mg g^{-1} FW (Figure 4.1 (c)).

In all three cultivars the concentration of quercetin glycosides and proanthocyanidins changed significantly during fruit development with each cultivar showing different patterns of change. In 'Golden Delicious' skin the flavonol levels dropped by about 50% from early to mid-season and then increased steadily to maturity returning almost to the level in the immature fruit. The pattern was slightly different in 'Granny Smith' apples where the flavonol concentration was higher in young fruit, and then decreased significantly from December to April. There was a similar pattern of change for the proanthocyanidin concentration. This drop in levels was particularly prominent in 1990/91, where quercetin glycosides and proanthocyanidins decreased by about 50% between December and April. However, there was a slight increase in the latter part of the season although this was followed by a further drop for the flavonols but not the proanthocyanidins at maturity. In 'Splendour' apples, concentrations of quercetin glycosides and proanthocyanidins decreased during the season, but they both increased from mid-March onwards along with the anthocyanins. In 1989/90 an end of season decrease in quercetin glycosides occurred, but in 1990/91 the last two sampling dates were possibly too far apart to detect a small peak if it did occur. Analysis of variance showed that the three cultivars were significantly different at the 5% level. However for 'Granny Smith' and 'Splendour' the differences between the two years were not significantly different for each of the cultivars. In 'Splendour' apples the concentration of anthocyanins was below 0.1 mg g^{-1} FW until early March when there was a rapid increase in levels until the final samplings in mid-April.

(a) 'Golden Delicious'



(b) 'Granny Smith'



(c) 'Splendour'

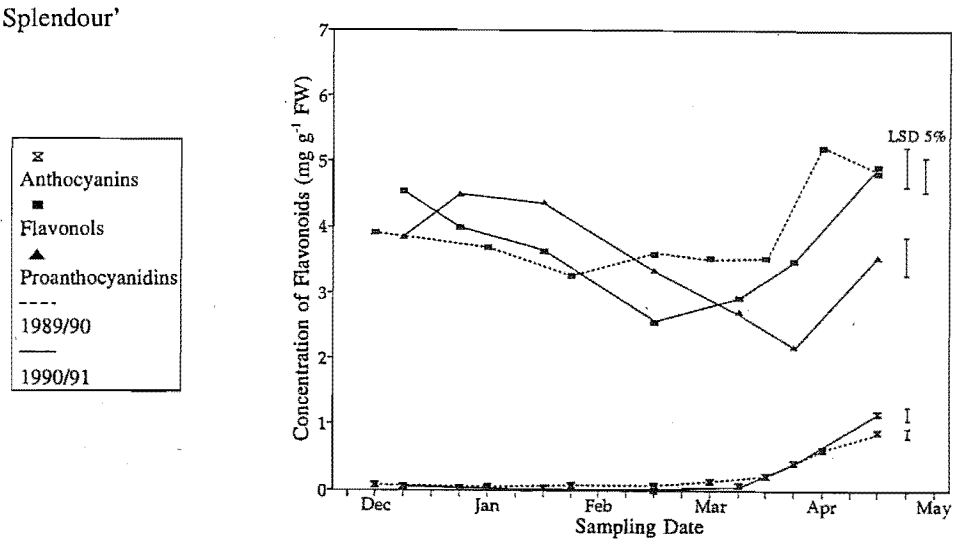


Figure 4.1: Developmental changes in concentrations of the three flavonoid groups in the skin of three apple cultivars ('Golden Delicious', 'Granny Smith' and 'Splendour') for 1989/90 and 1990/91 seasons (as determined by HPLC)

In 1989/90 anthocyanins reached a maximum of 0.9 mg g^{-1} FW and in 1990/91 the final level was 1.2 mg g^{-1} FW.

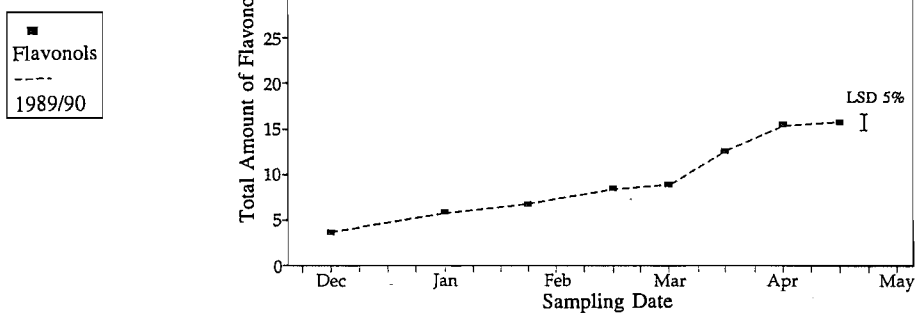
Although the concentration of quercetin glycosides and proanthocyanidins in the skin decreased during the season their total amount per fruit increased steadily as the fruit surface area increased (Figure 4.2). Linear regressions of the log [amounts] of quercetin glycosides and proanthocyanidins against time were computed and showed no significant differences between the two years, being similar for both cultivars, 'Granny Smith' and 'Splendour', over the whole season. Rates of increase were parallel, but with significantly different intercepts on the 'y' axis (Figure 4.2). Cyanidin glycosides in 'Splendour' showed a significantly different pattern of accumulation to the other flavonoid groups, being negligible until early March then rising rapidly.

Final amounts of quercetin glycosides were 16 mg for 'Golden Delicious' fruit, 33 mg (1989/90) and 28 mg (1990/91) for 'Granny Smith' fruit, and 44 mg (1989/90) and 42 mg (1990/91) for 'Splendour' fruit. Proanthocyanidins reached a final level of 17 mg and 31 mg for 'Granny Smith' and 'Splendour' respectively. Assuming synthesis, but not breakdown, this corresponds to the synthesis of 0.1 mg day^{-1} of quercetin glycosides in 'Golden Delicious', 0.18 mg day^{-1} for 'Granny Smith' and 0.3 mg day^{-1} for 'Splendour'. Proanthocyanidin synthesis was calculated at 0.1 mg day^{-1} for 'Granny Smith' and 0.21 mg day^{-1} for 'Splendour'. Cyanidin glycoside synthesis was 0.21 mg day^{-1} for 'Splendour' from mid-March onwards.

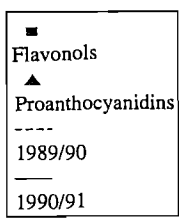
Relative amounts of quercetin glycosides

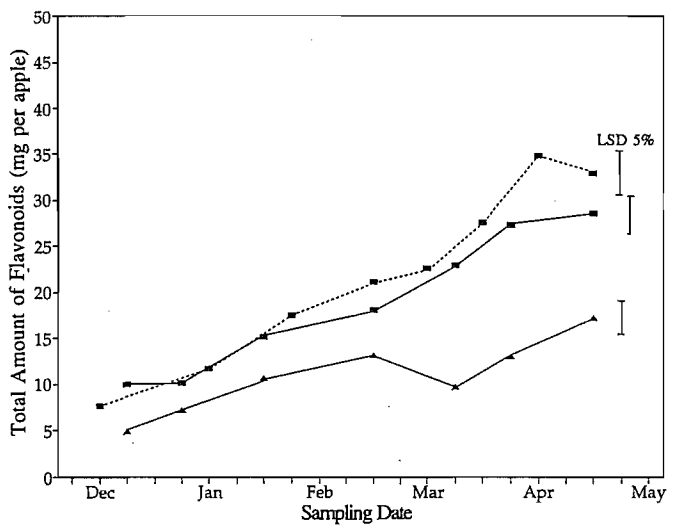
The percentage of each quercetin glycoside relative to total quercetin glycosides was calculated for 'Granny Smith' and 'Splendour' for both years and for 'Golden Delicious' for one year. All three cultivars synthesized the same quercetin glycosides, although the relative proportions differed to some extent. For 'Golden Delicious' the three major glycosides, comprising about 75% total glycosides were, in descending order, quercetin-3-galactoside, -3-arabinofuranoside, and -3-rhamnoside. The minor glycosides, quercetin-3-glucoside and -3-xyloside, together comprised about 15% of the total. The final 10% comprised those compounds present in trace amounts, namely quercetin-3-rhamnoglucoside, -3-arabinopyranoside and an unidentified quercetin glycoside. For 'Granny Smith' (Figure 4.3 (a)) the three major quercetin glycosides were present in the same order although the total composition was slightly lower at 70%. The ranking was the same for both years, although in 1989/90 quercetin-3-galactoside and -3-rhamnoside exhibited a slightly higher percentage of the total. The minor glycosides were the same but were present in a much higher proportion comprising 25% of the total while those present in trace amounts were only 5%. In 'Splendour' (Figure 4.3 (b)) the three main glycosides, comprising about 75% of the total glycosides, were similar to the other two cultivars except that the -3-rhamnoside was present in a higher proportion than -3-arabinofuranoside. The ranking was the same for both years. The ranking of the minor and trace glycosides was very similar to the other two cultivars and they accounted for 25%

(a) 'Golden Delicious'

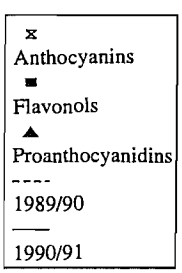


(b) 'Granny Smith'





(c) 'Splendour'



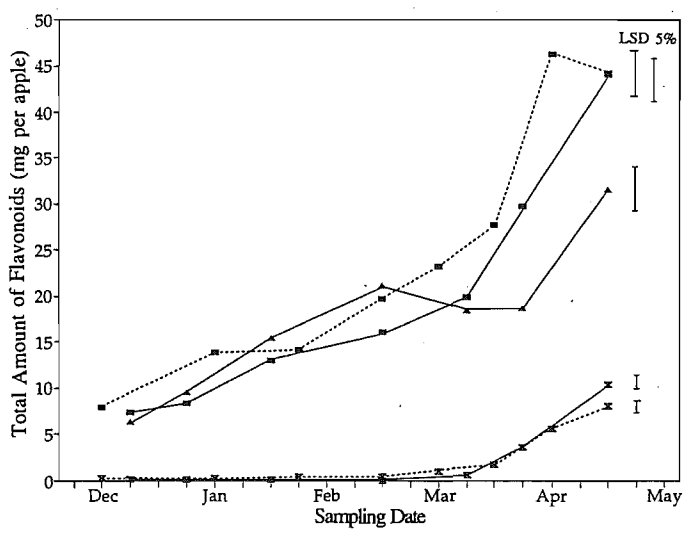
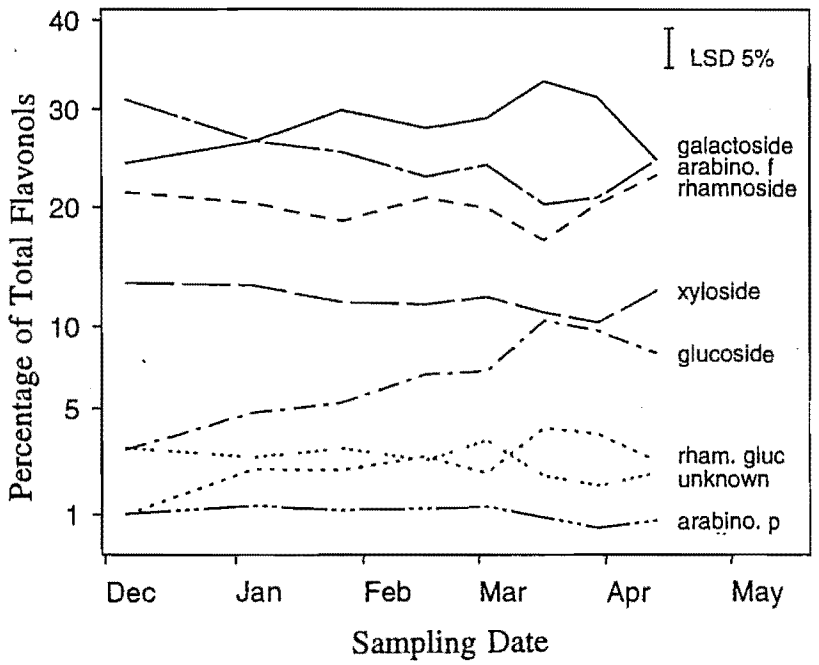


Figure 4.2: Developmental changes in the amounts of the flavonoid groups per apple for three apple cultivars ('Golden Delicious', 'Granny Smith' and 'Splendour') for 1989/90 and 1990/91 seasons (as determined by HPLC)

(a) 'Granny Smith'



(b) 'Splendour'

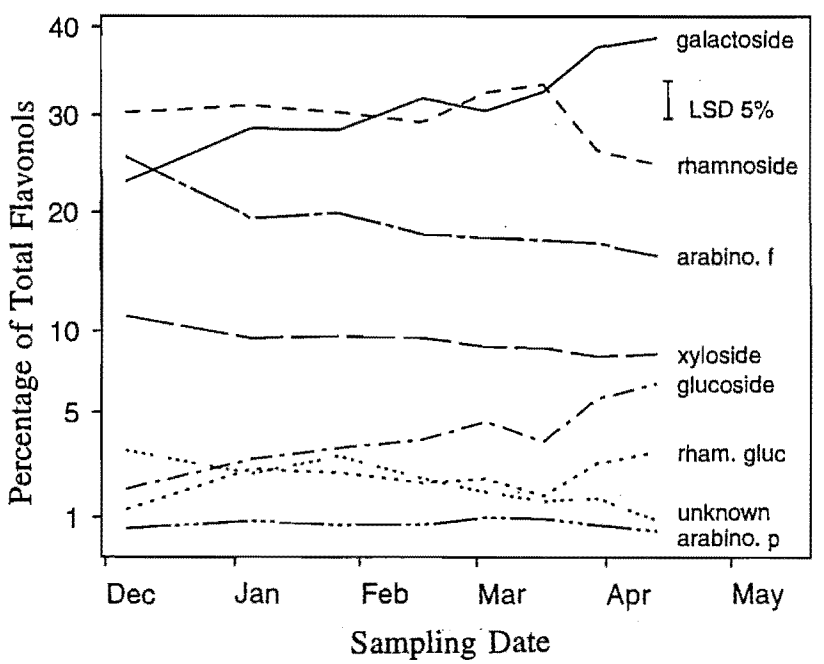


Figure 4.3: Developmental changes in individual quercetin glycosides for two apple cultivars ('Granny Smith' and 'Splendour') for the 1989/90 season (as determined by HPLC)

and 5% of the total respectively.

There were fluctuations in the proportions of the individual quercetin glycosides during the growth of the apple fruit, particularly at the end of the season. Quercetin-3-glucoside increased by about 8% at the end of the season for 'Splendour' in both years and 'Granny Smith' in 1990/91. However, in 1989/90 for 'Granny Smith' and 'Golden Delicious' there was a gradual rise in the proportion of quercetin-3-glucoside over the season. For both years 'Splendour' showed an end of season increase in quercetin-3-galactoside and a corresponding decrease in the relative proportion of quercetin-3-rhamnoside. There was no such change in either of these components in 'Golden Delicious' or 'Granny Smith'. Apart from these changes the proportions of the other compounds remained relatively constant.

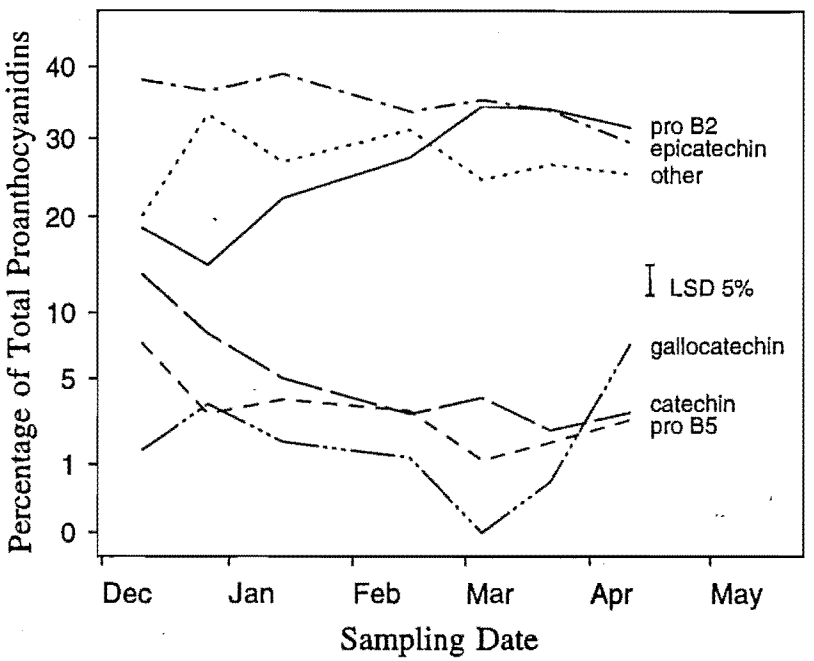
Relative amounts of proanthocyanidins

As with the quercetin glycosides, the percentage of each proanthocyanidin was calculated relative to the total. The main proanthocyanidins for both 'Granny Smith' and 'Splendour' were (-)-epicatechin and one of its dimers, procyanidin B2, which together comprised 60-65% of the total. (+)-Catechin, putative (+)-gallocatechin and procyanidin B5 (another (-)-epicatechin dimer) were only minor components comprising 5-15%. In both cultivars the proportion of unidentified proanthocyanidins reached 25-30%, although since this comprised several compounds individually each component in this total accounted for less than 5%. Proanthocyanidin profiles were similar for both cultivars, and there were significant changes in both during the season (Figure 4.4). (-)-Epicatechin was the predominant compound in both cultivars at the start of the season. However, in 'Granny Smith' procyanidin B2 increased significantly during development and was slightly higher than (-)-epicatechin at the end of the season, although the difference was not significant (Figure 4.4 (a)). In 'Splendour' there was also a rise in the percentage of procyanidin B2 but only until March after this there was a decrease however the final percentage was still higher that in the juvenile fruit (Figure 4.4 (b)). (+)-Catechin levels showed a significant change in both cultivars, dropping by about 75% over the season. (+)-Gallocatechin was proportionately highest in December and in April, being lower in the middle of the season. In 'Splendour' there was a slow but significant rise in the level of other proanthocyanidins over the season, this increase was not attributed to a single compound but a rise in several components. In 'Granny Smith' these levels fluctuated rather than showing any particular trend of change.

Changes in chlorophyll levels

Analysis of variance showed that the chlorophyll levels in all three cultivars were significantly different. At maturity 'Granny Smith' showed the highest levels at 170.8 $\mu g\ g^{-1}$ FW followed by 'Splendour' with 44.4 $\mu g\ g^{-1}$ FW and the 'Golden Delicious' at 34.9 $\mu g\ g^{-1}$ FW. The total concentration of chlorophyll decreased significantly during the development of the fruit in all three cultivars (Figure

(a) 'Granny Smith'



(b) 'Splendour'

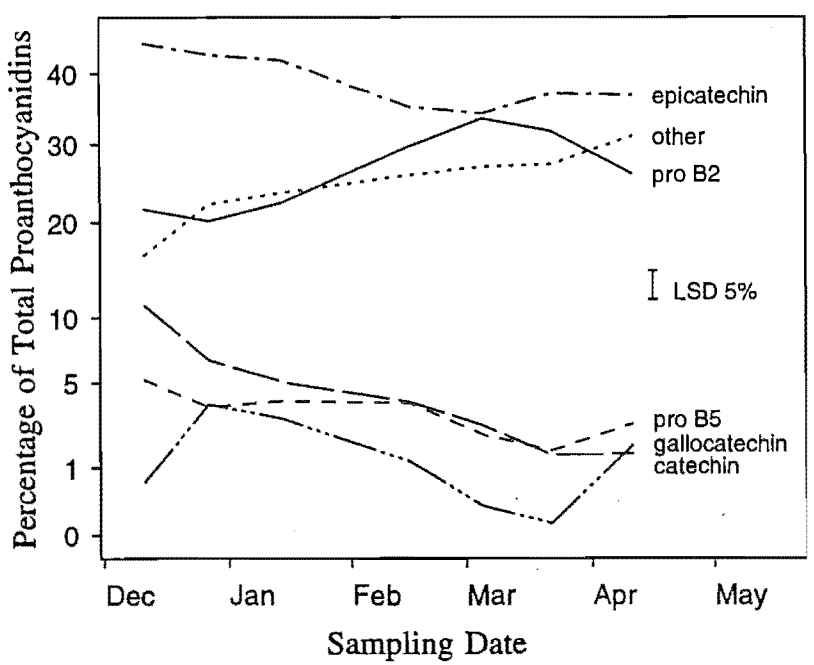


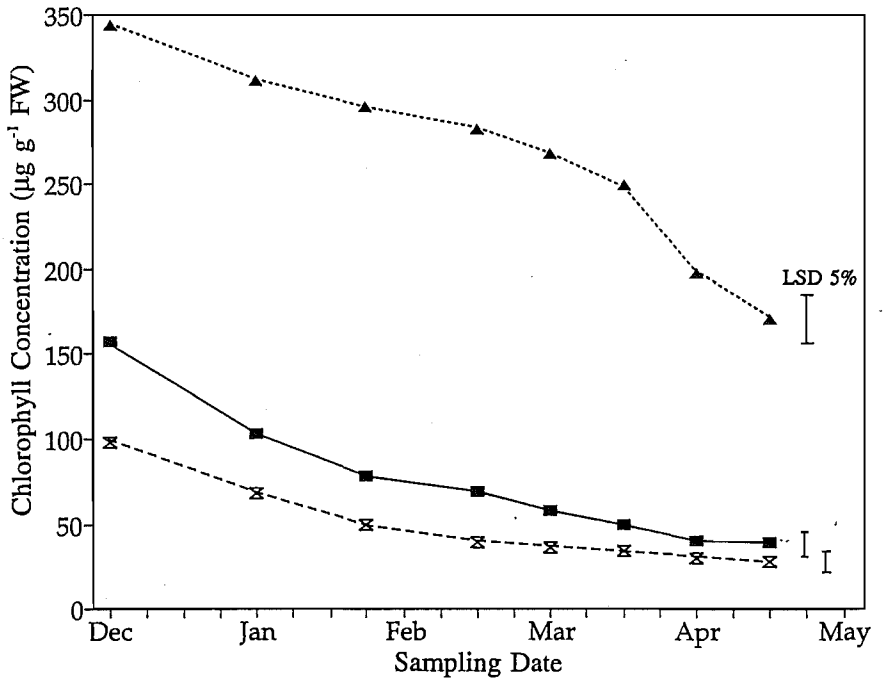
Figure 4.4: Developmental changes in individual proanthocyanidins for two apple cultivars ('Granny Smith' and 'Splendour') for the 1990/91 season (as determined by HPLC)

4.5 (a)). 'Splendour' showed the greatest decrease dropping 75% over the period measured, followed by 'Golden Delicious' (70%) and 'Granny Smith' (50%). However, the pattern of change was markedly different when calculated as the total amount of chlorophyll per fruit (Figure 4.5 (b)). In the cultivar 'Golden Delicious' there was a significant increase in chlorophyll level up to mid-February and then no significant change through to the end of sampling in mid-April. The pattern for 'Granny Smith' was different with a significant rise in chlorophyll levels through to mid-March which then dropped significantly to the end of the season. In 'Splendour' the period of chlorophyll increase was shorter only going until early January and after that point there was no significant change in levels.

Changes in carotenoids

Carotenoid levels were also significantly different for each of the cultivars. Despite being a yellow cultivar 'Golden Delicious' had the lowest carotenoid levels at maturity at 6.9 $\mu g\ g^{-1}$ FW. 'Granny Smith' had the highest level at 21.7 $\mu g\ g^{-1}$ FW and 'Splendour' contained 9.0 $\mu g\ g^{-1}$ FW. As with chlorophyll, the concentration of carotenoids decreased significantly during fruit development in all cultivars (Figure 4.6 (a)). Over the whole sampling period the decrease was similar to the chlorophylls being greatest for 'Splendour' (65%) then 'Golden Delicious' (60%) and 'Granny Smith' (50%). Again, as with chlorophyll, the pattern of change was different when expressed in terms of total carotenoids per apple (Figure 4.6 (b)). For 'Golden Delicious' there was a significant increase in carotenoids up to mid-February followed by a slight drop and then no significant change from March through to the end of sampling in mid-April. In 'Splendour' this pattern of change was not significantly different although the actual levels of carotenoids were different. The pattern of change in levels for 'Granny Smith' was the same as for the chlorophyll levels, with a significant rise through to mid-March when the level dropped significantly to the end of the season.

(a) Concentrations



(b) Amounts per apple

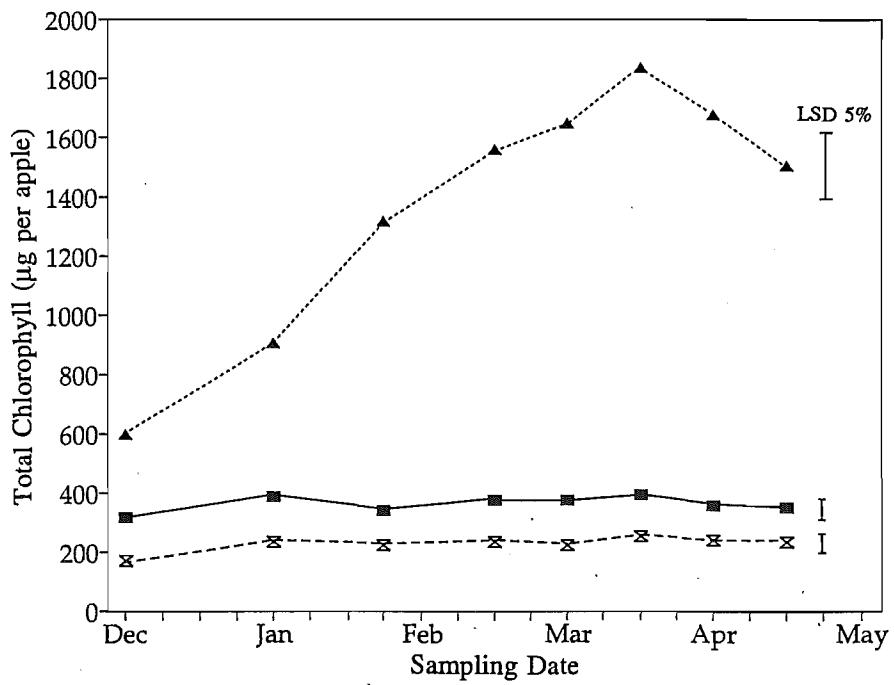
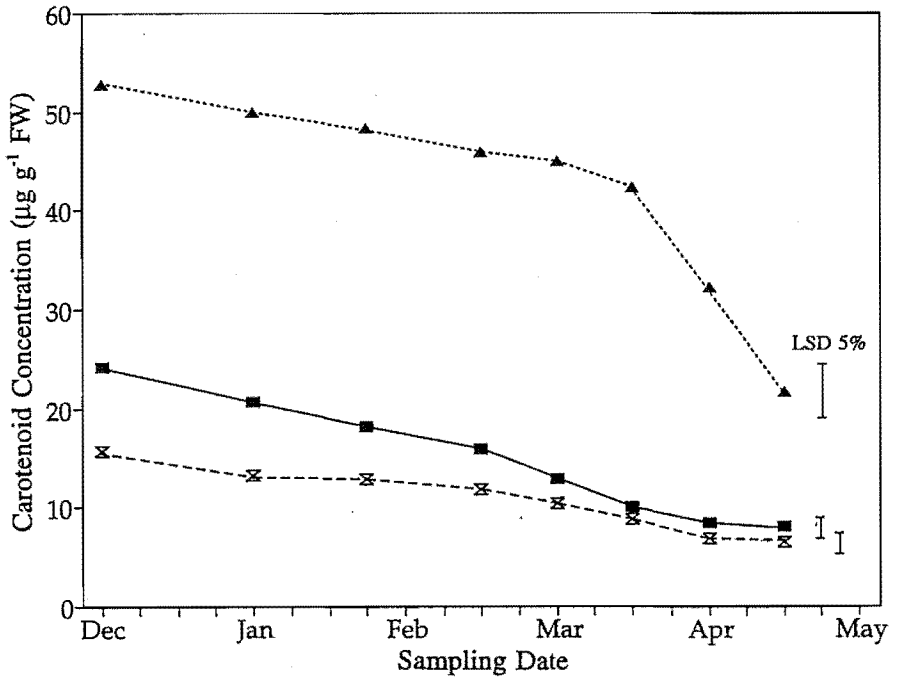
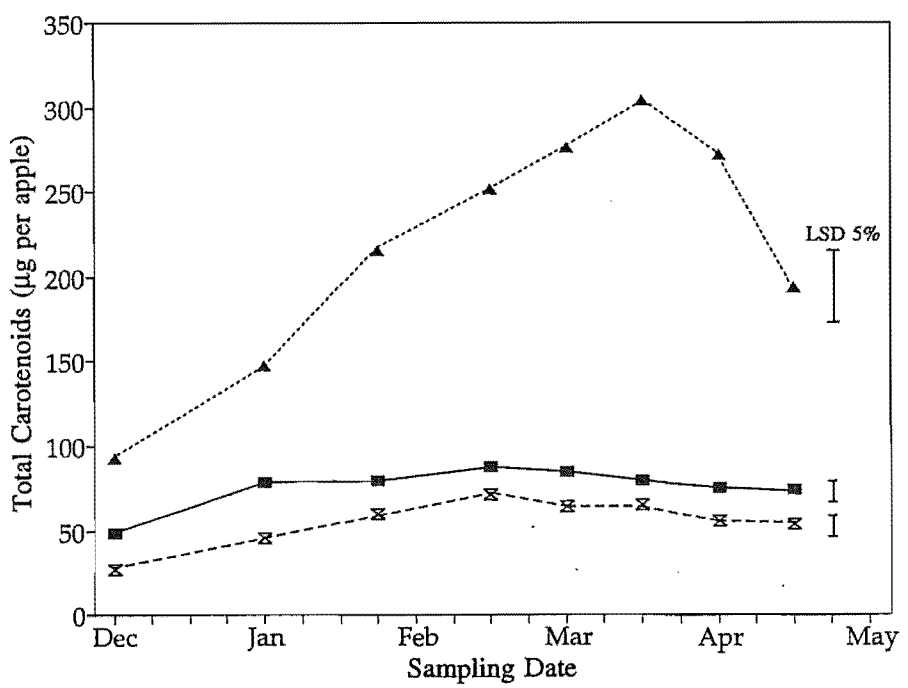


Figure 4.5: Developmental changes in chlorophyll levels for three apple cultivars ('Golden Delicious', 'Granny Smith' and 'Splendour') in the 1989/90 season

(a) Concentrations



(b) Amounts per apple



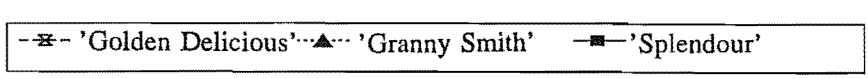


Figure 4.6: Developmental changes in carotenoid levels for three apple cultivars ('Golden Delicious', 'Granny Smith' and 'Splendour') in the 1989/90 season

4.4 Discussion

Developmental changes in the flavonoids

The flavonoid pigments present in immature fruit were identical to those found at maturity. The redness of the immature fruit was due to cyanidin-3-galactoside and even though the levels were not as high as at maturity the fruit appeared quite dark. This was probably due to the modifying effect of the chlorophylls which were present at much higher levels in the juvenile fruit. A significant drop in concentration was not detected; this was partly due to the first sampling being after significant visual drop in red colour. Earlier sampling of the fruit was not practical due to their small size and the difficulty removing the skin from the flesh since, as discussed earlier, contamination of skin samples with flesh would give distorted results due to the pigment distribution. Workman (1963) reported that some of his flavonol measurements were distorted because of the varying amounts of pulp adhering to the skin.

The main difference in flavonoid composition between non-red apple cultivars ('Golden Delicious' and 'Granny Smith') and a red cultivar ('Splendour') was the presence of cyanidin-3-galactoside in the latter. All three cultivars contained the same precursor profile of quercetin glycosides and the proanthocyanidins were the same in 'Granny Smith' and 'Splendour'. This suggests that, in non-red fruit the enzyme(s) converting leucocyanidin to cyanidin, this complex has been termed "anthocyanidin synthase" by Stafford (1990), were either not present or active. However, these fruit do contain the gene(s) for this enzyme(s) since production of cyanidin-3-galactoside is observed in juvenile fruit and also under stress conditions such as high light; thus, it is a matter of control. This control could be regulation of enzyme activity, enzyme inactivation or degradation of the anthocyanidin due to lack of glycosylation. Chalmers *et al.* (1973) postulated that the increase in anthocyanin concentration in mature fruit as compared with immature fruit was not based on an increased endogenous rate of synthesis towards maturity but that the fundamental difference lay in the potential to degrade anthocyanin. His theory was that anthocyanin was degraded in immature fruit skin as rapidly as it was formed whereas after maturity the rate of degradation was lower than the rate of synthesis. Later, Faragher (1983) stated that the importance of degradation with respect to regulation of anthocyanin levels was unknown. Further investigation of the activity of some of the enzymes of the flavonoid biosynthetic pathway in Section Five may shed some light on this. Proctor (1974) reported changes in the energy requirement for anthocyanin synthesis during the development of the fruit and this is another possible explanation for these changes since as the fruit ripen less energy is required for synthesis of other compounds thus resulting in higher anthocyanin biosynthesis.

Although both cultivars contained the same quercetin glycosides, there were differences between the relative proportions of individual glycosides. In 'Granny Smith' and 'Golden Delicious' quercetin-3-galactoside and 3-arabinofuranoside were the principal glycosides, with the 3-rhamnoside a little lower in concentration than arabinofuranoside. In 'Splendour' quercetin-3-rhamnoside was present in higher amounts than the 3-arabinofuranoside but quercetin-3-galactoside was still the predominant glycoside.

However, there were no significant changes in the proportions of the glycosides during the season. This stability of the quercetin glycosides suggests either coordinate regulation of glycosyltransferases in apple fruit or common glycosylation enzyme(s) with different sugar specificities.

The concentration of quercetin glycosides and proanthocyanidins was highest in early season but had decreased significantly by mid season for 'Granny Smith' and 'Splendour'. The highest concentrations of proanthocyanidins were reported for young fruit by Burda *et al* (1990) and Mosel and Herrmann (1974a). In 'Splendour' there was an increase in concentration of all flavonoids during maturation. (+)-Gallocatechin was a very minor component of the total proanthocyanidins during mid-season, but at the end of the season, increased to about 8% in Granny Smith and 3% in 'Splendour'. Mosel and Herrmann (1974a) also reported an end-of-season increase in concentration of (+)-gallocatechin.

As expected the anthocyanin concentration increased during ripening which is in agreement with previous studies (Creasy, 1968b; Knee, 1972; Chalmers *et al.*, 1973). However the changes in flavonols and proanthocyanidins reported here differ from those seen previously. Past reports on the concentrations of flavonols noted that they remained constant or dropped during ripening (Workman, 1963; Gorski & Creasy, 1977; Dick, 1986). Although, in agreement with Workman (1963) there was an increase in the amount of flavonoids per fruit during development. The changes in concentration of the proanthocyanidins reported here also differ slightly from what has previously been reported, since a decrease was noted during fruit development. Although this has been reported previously (Mosel & Herrmann, 1974a; Burda *et al.*, 1990) the increase in total levels during ripening has not been reported. The reasons for these differences are unclear but it may be varietal, or possibly due to environmental influences or cultural practices, as has been discussed previously with regard to flavonoid levels.

The parallel changes in concentration of quercetin glycosides, proanthocyanidins, and cyanidin glycosides during fruit growth and maturation of both cultivars suggests a coordinate regulation of flavonoid enzymes in response to common environmental and/or developmental signals. These changes are examined further in Section Five where some of the enzymes of the flavonoid pathway were studied and changes in activity related to changes in pigment concentration. From the pattern of pigment accumulation it is likely that there could be high levels of enzyme activity in the immature fruit and again at ripening. The level of enzyme activity during a large part of the growth of the fruit is likely to be lower, although there must still be some activity since the total amount of flavonoids per fruit increased throughout development.

The rates of synthesis of the flavonoids were significantly different between cultivars varying from 0.1 mg day^{-1} for 'Golden Delicious' (flavonols only), to 0.28 mg day^{-1} for 'Granny Smith' and 0.51 mg day^{-1} for 'Splendour', which increased to 0.72 mg day^{-1} from mid-March with the synthesis of the anthocyanins. Whether these differences are due to differences in rates of synthesis, enzyme activity and inhibitors, or other factors such as degradation, cannot be determined from this data.

Since there were no significant differences in the pattern of change within a cultivar between years this tends to indicate endogenous control of the developmental changes rather than as a result of

environmental stimuli. Environmental factors, such as temperature and sunshine hours, were different in each season. Thus, if these were the major influencing factors on developmental changes significant differences in the pattern of change would be expected in different seasons. Although endogenous signals probably control the pattern of change it is likely that environmental factors do influence flavonoid levels to some extent.

Developmental changes in the plastid pigments

All three apple cultivars showed chlorophyll concentrations in the skin decreased during development to varying degrees, which is in agreement with that reported previously for a number of cultivars (Workman, 1963; Knee, 1972; Gorski & Creasy, 1977). These changes have not expressed previously in terms of amounts per apple and it appears that in 'Golden Delicious' and 'Splendour' significant synthesis of chlorophyll occurred in the early stages of development resulting in an increase in levels. After this the levels remained constant which may have been due either to a decrease in synthesis or an increase in the rate of degradation. In 'Granny Smith' the period of high chlorophyll synthesis continued for much longer than the other two cultivars, continuing until one month prior to maturity but it was followed by a significant drop, presumably due to a big increase in the rate of degradation.

The decrease in carotenoid concentration in all three cultivars throughout the season differs from that reported by many other workers (Gorski & Creasy, 1977; Gross *et al.*, 1978). However Mussini *et al.* (1985) also reported a drop in carotenoids during the development of 'Granny Smith' fruit. Although other researchers have noted a drop in carotenoids during the early stages of development this has often been followed by a rise which was not noted in this study. As with the chlorophyll levels, carotenoids have not been expressed previously in terms of amounts per apple. The patterns followed were very similar to those of chlorophyll indicating a similar control mechanism. This could be an environmental factor such as light or temperature which are well known to influence synthesis and degradation of these pigments (Britton, 1988; Rüdiger & Schoch, 1988) and action of degrading enzymes. Otherwise the levels may be under endogenous control or a combination of both endogenous and environmental stimuli.

In 'Granny Smith' apples the levels of chlorophyll remained high at maturity and there were also high levels of carotenoids but they were masked by the high chlorophyll; this resulting in the typical bright green appearance of the fruit. On the other hand, 'Golden Delicious' fruit had much lower levels of chlorophyll, which meant that the carotenoids were 'unmasked' during ripening, giving the fruit its characteristic yellow colour even though the levels of carotenoids were not exceptionally high. In 'Splendour' the chlorophylls also dropped to levels that had limited influence on fruit colour and did not darken the red appearance of the fruit. Carotenoid content was slightly above the levels reported by Gorski & Creasy (1977) making the apple fruit appear yellow. Thus the carotenoids may have had some influence on the final appearance making the red colour due to anthocyanins tend towards orange-red

rather than blue-red.

Thus, changes in plastid pigments have a significant influence on the final appearance of the fruit. The inbuilt starting level of the plastid pigments also would appear to have a significant effect. For example if chlorophyll levels are very high in immature fruit, such as 'Granny Smith', it is unlikely that they will drop low enough to 'unmask' the carotenoids and will make the fruit appear green. Conversely, if the initial chlorophyll levels are low, then it is likely that they will drop further to reveal the yellow of the carotenoids. Fruit appearance is thus very much a function of the endogenous plastid pigment levels and their rate of degradation (or turnover).

4.5 Conclusions

The composition of the flavonoids in all three cultivars was similar with the only difference being that of 'Splendour' which synthesized cyanidin glycosides during ripening which suggests that the main control point in anthocyanin biosynthesis is at the final stages in the pathway, involving the conversion of leucocyanidin (flavan-3,4-diol) to cyanidin. The general pattern of change in the concentration of the flavonols and proanthocyanidins was a drop from high levels in young fruit through to the middle of the season. This was followed by a rise in concentration although there were significant differences between the cultivars but not between years. This could mean that there are likely to be high levels of enzyme activity in the immature fruit and the mature fruit just before harvest with low levels in the middle. Although there were significant changes in concentration the relative proportions of the major quercetin glycosides and proanthocyanidins were stable during fruit development. This suggests either coordination of the glycosylation enzymes or a common glycosylation enzyme(s) with differing sugar specificities. For 'Splendour' the increase in quercetin glycosides and proanthocyanidins correlated with cyanidin glycoside synthesis and this suggests a coordinate regulation of the enzymes of the flavonoid biosynthetic pathway during fruit development. There were significant differences between the three cultivars in the total amounts of flavonoids synthesized on a daily basis indicating possible differences in enzyme activity. The total amounts of flavonoids per fruit increased throughout the season as the fruit's surface area increased showing that synthesis of the flavonoids was taking place throughout fruit development.

The concentration of both carotenoids and chlorophyll decreased significantly during fruit development in all three cultivars but the total amount per apple increased for all cultivars during early development. The patterns of change in chlorophyll and carotenoid levels were similar for 'Golden Delicious' and 'Splendour' but significantly different for 'Granny Smith'. When comparing within a cultivar the same pattern of change occurred for both chlorophyll and carotenoids suggesting similar control mechanisms.

The final appearance of the fruit is the result of the initial pigment concentrations plus the combined changes and hence the resultant final concentration of all three pigment groups (flavonoids, carotenoids and chlorophyll). The yellow of 'Golden Delicious' resulted from the unmasking of carotenoids due to the decrease in chlorophyll unlike 'Granny Smith' in which, although the chlorophyll level decreased, it remained high so that the fruit still appeared green at maturity. 'Splendour' differs in that it synthesises anthocyanins and the brightness and clarity of its red colour was due to the disappearance of most of the chlorophyll which would otherwise have given the fruit a bronze appearance.

Section 5 - BIOSYNTHESIS OF THE FLAVONOIDS

5.1 Introduction

5.1.1 The flavonoid biosynthetic pathway

The main pathway of flavonoid biosynthesis was deduced some 20 years ago using data from chemicogenetical studies (Harborne, 1962) and from the results of feeding radioactively labelled precursors (Grisebach, 1965). In the past few years considerable progress has been made in elucidating the biosynthesis of flavonoids and, in particular, knowledge of the enzymology has developed rapidly. With the exception of the anthocyanins, where a few reactions still remain unknown, the essential steps of the biosynthetic pathway of the main flavonoid classes are now established.

Precursor biosynthesis

All flavonoids derive their carbon skeletons from two basic compounds: malonyl-CoA and the CoA ester of a hydroxycinnamic acid, generally coumaroyl-CoA. The origins of the direct flavonoid precursors are shown in Figure 5.1, and the enzymes in Table 5.1. Both flavonoid precursors are derived from carbohydrates. Malonyl-CoA is synthesized from acetyl-CoA and carbon dioxide, the reaction being catalysed by acetyl-CoA carboxylase. The supply of 4-coumaroyl-CoA is more complex. It involves the shikimate/arogenate pathway, which is the main route to the aromatic amino acids phenylalanine and

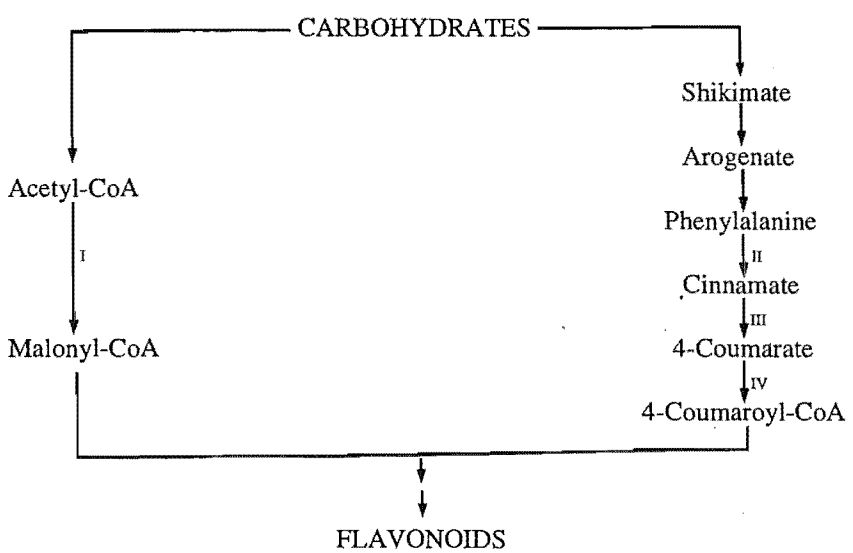


Figure 5.1: Biosynthetic origin of the flavonoid precursors

Table 5.1: The known enzymes leading to the various flavonoid classes (adapted from Heller & Forkmann, 1988 and Harborne, 1988)

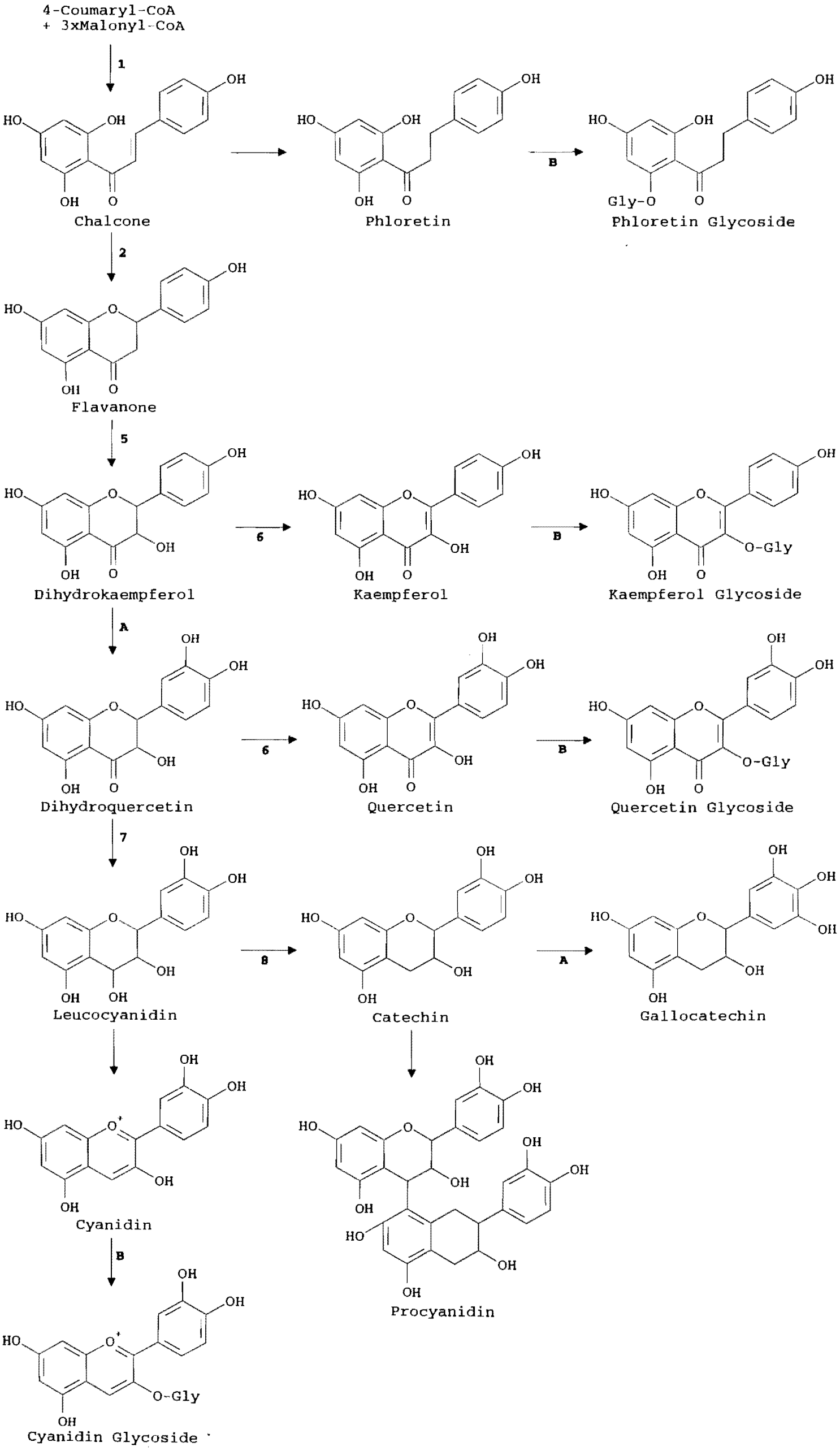
Enzymes	EC number
Precursor biosynthesis	
I Acetyl-CoA carboxylase	6.4.1.2
II Phenylalanine ammonia-lyase	4.3.1.5
III Cinnamate 4-hydroxylase	1.14.13.11
IV 4-Coumarate:CoA ligase	6.2.1.12
Synthesis of flavonoid classes	
1 Chalcone synthase	2.3.1.74
2 Chalcone isomerase	5.5.1.6
3 2-Hydroxyisoflavanone synthase	
4 Flavone synthase	
5 (2*S*)-Flavanone 3-hydroxylase	1.14.11.9
6 Flavonol synthase	
7 Dihydroflavonol 4-reductase	
8 Flavan-3,4-*cis*-diol 4-reductase	
9 Flavonoid 3-O-glucosyl-transferase	2.4.1.91
Flavonoid modification	
A B-ring hydroxylases	
B Glycosyl transferases	
C Methyl transferases	
D Acyl transferases	
E Sulphate transferases	

tyrosine in higher plants (Jensen, 1985). Subsequent transformation of phenylalanine to *trans*-cinnamate is catalysed by phenylalanine ammonia-lyase (PAL) which provides the link between primary metabolism and the phenylpropanoid pathway. PAL is often regarded as a key step in the biosynthesis of the flavonoids. Aromatic hydroxylation of cinnamate, by cinnamate 4-hydroxylase, leads to 4-coumarate which is further transformed to 4-coumaroyl-CoA, by action of 4-coumarate:CoA ligase. It is generally accepted that 4-coumaroyl-CoA is the main, or even exclusive, precursor for flavonoids but other hydroxycinnamic acids, such as caffeate, may be involved in rare cases (Heller & Forkmann, 1988).

Main pathway

Based on the elucidation of the flavonoid biosynthetic pathway in other plants, and the particular compounds present, a similar pathway can be postulated for the biosynthesis of flavonoids in apple skin (Figure 5.2). The major difference, from the general pathway proposed by Heller & Forkmann (1988), is that in apples some classes of the flavonoids, such as the flavones, isoflavonoids and pterocarpans are not synthesized.

Figure 5.2: Putative biosynthetic pathway for the flavonoids in apples


4-Coumaryl-CoA
+ 3xMalonyl-CoA
1
Chalcone
Phloretin
B
Phloretin Glycoside
2
Flavanone
5
Dihydrokaempferol
6
Kaempferol
Kaempferol Glycoside
A
Dihydroquercetin
Quercetin
Quercetin Glycoside
7
Leucocyanidin
8
Catechin
Gallocatechin
Cyanidin
Procyanidin
Cyanidin Glycoside

(a) Formation of the flavanone

The first committed step of flavonoid biosynthesis is the condensation of three molecules of malonyl-CoA with 4-coumaroyl-CoA to form the C_{15} intermediate 4,2′,4′,6′-tetrahydroxychalcone (naringenin chalcone). Formation of this chalcone is catalysed by the enzyme chalcone synthase (CHS) which has been well characterized and assayed for a number of plants (Ebel & Hahlbrock, 1982; Britsch & Grisebach, 1985). This enzyme is often regarded as a key enzyme of flavonoid biosynthesis and recent work has been undertaken on the mode of regulation (Heller & Forkmann, 1988; Stafford, 1990).

Most flavonoid classes (e.g. flavonols, anthocyanins) are derived from a flavanone intermediate which involves the stereospecific isomerization of the chalcone to a (2S)-flavanone, naringenin, which is catalysed by chalcone isomerase (CHI) (Harborne, 1988). Details of this step are discussed later. Flavanones represent a branch point in the biosynthetic sequence in many plants since they may be converted to either flavones or to isoflavones (Stafford, 1990). However, neither of these classes of compounds are observed in apples, whether this is due to lack of the enzymes responsible for the conversion, or channelling into other flavonoid classes has not been determined.

(b) Dihydrochalcone formation

Dihydrochalcones (e.g. phloretin) are probably derived from chalcones (e.g. naringenin chalcone) by reduction of the double bond in the central open carbon chain (Stafford, 1990). However, there has been no documentation of the enzyme involved in this step. The dihydrochalcone is subsequently glycosylated by a flavonoid glycosyltransferase to give compounds such as phloridzin.

(c) Flavanone 3-hydroxylase

The conversion of the flavanone to a dihydroflavonol was shown first in tracer studies and is catalysed by the enzyme flavanone-3-hydroxylase (Beggs *et al.*, 1986). This enzyme has been detected from a variety of plants and has been characterised as a 2-oxoglutarate dependent dioxygenase, requiring ferrous iron and ascorbate as cofactors (Britsch & Grisebach, 1986). The order in which 3- and 3′-hydroxylation occurs is not clear, but some data suggests that 3-hydroxylation occurs first in some plants (Stafford, 1990). In apples, flavones are not synthesised from the flavanone which may be due to either lack of flavone synthase, or higher activity and specificity of the flavanone 3-hydroxylase.

(d) Flavonol formation

The enzyme flavonol synthase, which converts dihydrokaempferol to kaempferol or dihydroquercetin to quercetin, is also a 2-oxoglutarate dependent dioxygenase but has not been studied in as much detail as many other enzymes. In a study on *Matthiola* (Spribille & Forkmann, 1984) it was demonstrated that enzyme activity in flowers related to their flavonol content. Gerats *et al.* (1982) found that the substrate specificity of the enzyme related to the composition of the flavonols. Thus it is likely

that the flavonol synthase in apples would have specificity towards 3',4'-hydroxylated compounds, such as dihydroquercetin rather than dihydrokaempferol.

(e) Catechin and proanthocyanidin formation

The first evidence that leucoanthocyanidins (or flavan-3,4-diols) were intermediates in proanthocyanidin biosynthesis was obtained when dihydroquercetin was converted into *cis*-leucocyanidin by a preparation from *Pseudotsuga menziesii* cell suspension culture (Stafford & Lester, 1982). This conversion was catalysed by the enzyme dihydroflavonol reductase (DHFR). The further reduction of leucoanthocyanidins to catechins (flavan-3-ols) has also been shown to be catalysed by a reductase, flavan-3,4-*cis*-diol 4-reductase (Harborne, 1988). The final step in proanthocyanidin biosynthesis, in which catechins are linked together or to leucoanthocyanidins, may occur spontaneously via a carbocation or quinone-methide intermediate or may be under enzymic control. No evidence has yet been obtained for the existence of a condensing enzyme for producing the polymeric proanthocyanidins. However, the presence of one or more of these enzymes is very probable for regulatory reasons (Stafford, 1990). Different proanthocyanidin patterns are observed in different plants but within a species the patterns are usually similar so there must be some mechanism to control this.

(f) Anthocyanin formation

Tracer studies with seedlings and cell suspension cultures and supplementation studies demonstrated that dihydroflavonols were intermediates in anthocyanin biosynthesis (Stafford, 1990). Subsequently the involvement of leucoanthocyanidins in anthocyanin synthesis has been demonstrated by a series of supplementation experiments in which these precursors were fed to acyanic flowers and anthocyanin production was initiated (Heller *et al.*, 1985). This has also been observed in *Camellia* flowers (Lynn Bowen, pers. comm.). Although proanthocyanidins give rise to their respective anthocyanidins when heated in acid, there is no evidence for this conversion *in vivo*, even during senescence (Ishikura, 1972). However, the enzymic conversion of leucocyanidin to anthocyanidin has not yet been achieved although various possible reaction schemes have been postulated (Heller & Forkmann, 1988; Stafford, 1990). Stafford (1990) postulated a tight enzyme complex termed "anthocyanin synthase". This complex catalyses a number of steps which involve 2-hydroxylation, dehydration and glycosylation of the leucoanthocyanidin. Genes involved in anthocyanin biosynthesis have now been isolated (Gutterson, 1993) and these may provide information on the enzymes involved.

Since anthocyanidins do not normally occur in plant tissues, and since neither dihydroflavonols nor leucocyanidins are glycosylated, it is assumed that the final step in flavonoid biosynthesis is glycoside formation (Heller & Forkmann, 1988). Details of the glycosylation step are discussed later.

Of all these enzymes involved in flavonoid biosynthesis only two have been reported in apples, these are PAL (Tan, 1979; Arakawa *et al.*, 1986; Kubo *et al.*, 1988) and a flavonoid glucosyltransferase (Macheix *et al.*, 1981). Thus there is still a lot of work to do on the flavonoid enzymes to determine how

the presence and levels of enzyme activity relate to the composition and concentration of the flavonoids in apple skin. The rate-limiting step also needs to be determined as this may be important for the future genetic manipulation of colour.

B-Ring hydroxylation

The 4′-hydroxyl group of the B-ring is introduced by incorporation of 4-coumaroyl-CoA during synthesis of the flavonoid skeleton. The B-ring substitution pattern may be determined in two ways: (1) incorporation of already substituted hydroxycinnamic acid derivatives during synthesis of the C_{15} skeleton, or (2) substitution at the C_{15}-stage by specific hydroxylases (Heller & Forkmann, 1988). In the first case 4-coumarate would be the precursor for 4′-hydroxy and caffeate for 3′,4′-hydroxyflavonoids. In the second case 4-coumarate would be the precursor for all the differently substituted flavonoids with modification occurring at a later stage.

For all chalcone synthases tested so far, 4-coumaroyl-CoA was the best substrate which suggests that the second hypothesis is more likely (Harborne, 1988). However, some chalcone synthases do show *in vitro* activity towards caffeoyl-CoA and feruloyl-CoA, with the formation of eriodictyol and homoeriodictyol chalcones respectively but it is doubtful whether these reactions normally occur *in vivo*, except in a few species (Heller & Forkmann, 1988). There is genetic and enzymic evidence, in *Verbena hybrida* (Stotz *et al.*, 1984) and *Silene pratensis* (Kamsteeg *et al.*, 1980), that chalcone synthase may utilize caffeoyl-CoA as a substrate, but only as a minor route. Thus, most of the accumulated evidence favours the second route with substitution, at the later C_{15}-stage by specific hydroxylases.

The enzymes flavonoid 3′-hydroxylase and flavonoid 3′,5′-hydroxylase have now been isolated from a number of plant sources (Stotz & Forkmann, 1982; Hagmann *et al.*, 1983). They both behave like a monooxygenase in their cofactor requirements, needing NADPH and molecular oxygen (Harborne, 1988). The enzyme flavonoid 3′-hydroxylase gives rise to flavonoids with 3′,4′-hydroxylation, such as quercetin, and it may catalyse a number of different substrates (Table 5.2). It was first demonstrated in microsomal preparations of *Haplopappus* cell cultures (Fritsch & Grisebach, 1975) and has since been found in microsomal fractions prepared from a range of flower and seedling tissues (Heller & Forkmann, 1988). Flavonoid 3′-hydroxylase is found in plants with a dominant allele for cyanidin production and is absent from recessive genotypes which make pelargonidin (Heller & Forkmann, 1988). Further evidence that the 3′-hydroxyl group is inserted in the flavonoid nucleus is obtained from information on the genes involved in 3′-hydroxylation. These genes exert no influence on substrate specificity of the chalcone synthase (Spribille & Forkmann, 1981 & 1982). One characteristic of these hydroxylases is that they are non-specific in the hydroxylation pattern of the C-ring. This provides a pattern often referred to as a "grid" in which dihydroquercetin can be produced by two pathways from naringenin, via either eriodictyol or dihydrokaempferol (for details see Stafford, 1990).

The enzyme flavonoid 3′,5′-hydroxylase gives rise to flavonoids with the 3′,4′,5′-hydroxylation pattern such as myricetin. It can catalyse a number of reactions converting naringenin and to 5,7,3′,4′,5′-

Table 5.2: Enzymes of flavonoid hydroxylation

Enzyme	Reactions catalysed	Plants and reference
Flavonoid 3′-hydroxylase	Naringenin→eriodictyol Dihydrokaempferol→dihydroquercetin Kaempferol→quercetin Apigenin→luteolin	Many spp., e.g. Parsley (Hagmann *et al.*, 1983)
Flavonoid 3′,5′-dihydroxylase	Naringenin→5,7,3′,4′,5′-penta OH flavanone Dihydrokaempferol→dihydromyricetin Dihydroquercetin→dihydromyricetin	Some spp. e.g. *Verbena* (Stotz & Forkmann, 1982)

pentahydroxyflavanone and dihydrokaempferol to dihydromyricetin. The same enzyme also converted eriodictyol and dihydroquercetin to the same products, and can hydroxylate at the flavone level (Stafford, 1990).

Since apples contain predominantly 3′,4′-hydroxylated flavonoids the flavonoid 3′-hydroxylase would be expected to be present and have high activity. Small quantities of a 3′,4′,5′-hydroxylated flavonoid have also been noted, e.g. (+)-gallocatechin, so there must also be a 3′,5′-hydroxylase present although activity would be expected to be low. It would also be expected to exhibit limited substrate specificity since no other 3′,4′,5′-hydroxylated compounds are observed in apples.

5.1.2 Regulation of flavonoid biosynthesis

The most important control mechanisms in phenolic metabolism involve control of the amount of enzymes, regulation of enzyme activity, compartmentation of enzymes, availability of precursors and intermediates, and integration in the differentiation and development programs of the plant (Macheix *et al.*, 1990). In many plants flavonoids can be considered to be constitutive, their appearance being controlled by endogenous factors during normal development (Heller & Forkmann, 1988). However, flavonoid synthesis may also be induced, that is controlled by external factors such as light and stress. It is not clear if the pathways for constitutive and induced enzymes are the same and whether induced enzymes are less stable than constitutive ones (Stafford, 1990).

Control of a particular flavonoid pathway may be at several levels. One level controls the concentration of an enzyme by the relative rates of synthesis and degradation of the enzyme. In parsley cell cultures half-lives of the flavonoid enzymes range from 10 to 40 hours (Hahlbrock *et al.*, 1976). The decrease can be due to an irreversible degradation of the enzyme called "inactivation". This has been demonstrated in sliced potato tubers after light induction where control of PAL by this mechanism

regulates the flavonoid pathway (Zucker, 1968). Robbins & Dixon (1984) summarized two models of degradation, one for CHS in parsley and the other for CHI in bean.

At another level, the amount of flavonoid synthesised may be controlled by the actual amount of the enzyme that is active at any one time by the interaction of already formed enzyme with inhibitors and inactivating-activating agents. Enzymes, although present, may not be always active; inactivation can be reversible or irreversible. End product inhibition of some enzymes is possible although this has not been demonstrated clearly (Stafford, 1990). Increase in activity induced by light or stress may not be due solely to *de novo* synthesis but the result of conversion of inactive to active forms (Stafford, 1990). This has been shown with chalcone isomerase in *Phaseolus vulgaris* and *Glycine max* (Robbins & Dixon, 1984; Dixon *et al.*, 1988). The only example of control at this level that has been demonstrated in apple is PAL-IS, an inactivating system for phenylalanine ammonia-lyase that is discussed later.

A third level involves compartmentation of the enzymes. Enzyme activity can be controlled by its intracellular and tissue site relative to its substrates, by endogenous inhibitors and activators. There may also be organisation with other enzymes of a pathway into multienzyme complexes and sequences that channel intermediates directly from one protein to another in microcompartments (Stafford, 1990). All levels, affecting enzyme activity, can be affected by exogenous as well as endogenous factors.

To date all evidence suggests that phenolic biosynthesis is highly regulated and typically involves a coordinated short-term increase in expression of genes for individual pathway enzymes (van der Meer *et al.*, 1993). This results in bursts of synthesis of particular end products at particular stages in development of a plant or in response to stimuli from the environment (Rhodes, 1985). This degree of regulation in the pathway, and its receptivity towards a variety of factors, suggests strongly that phenolics play an important role in the metabolism of plants and their interaction with the environment.

Formation of anthocyanins in apples is genetically determined but may be influenced by various environmental factors as reviewed by Walter (1967). However, information is often conflicting on the way in which these factors act and the interaction between them is still disputed. Some cultivars suffer from poor colouration in certain seasons and regions. There can also be variation from tree to tree and even from fruit to fruit on the same tree. Study of the flavonoid enzymes, their changes during development (endogenous control) and in response to exogenous factors such as light, may yield information on the control mechanisms in apples.

5.1.3 Organisation of flavonoid pathways

Regulation of the flavonoid pathway by external agents is beginning to be understood but regulation by endogenous agents is relatively unknown (Stafford, 1990) and there are still many areas that are not completely understood such as the organisation of the pathway and the control of individual end products. Since one plant can synthesise more than one flavonoid end product in the same cell, organisation into unidirectional arrays of contiguous enzymes in a sequence or aggregate could be an important means of

regulation (Stafford, 1990). There are two general concepts of the organisation of flavonoid biosynthesis in the plant; an integrated pathway producing all the flavonoid classes, or several parallel pathways each leading to a single compound or class of compounds (Figure 5.3). Indirect evidence favours the concept of parallel pathways with competition for substrates occurring mainly at the entrance into the flavonoid pathway, or in earlier pathways, rather than for C_{15} intermediates in one metabolic pool. Thus, endogenous regulators would be expected to control the entire complex at least at the C_{15} level. Formation and maintenance of the organised complex rather than the initial enzyme chalcone synthase, or its substrates, would be the rate-limiting step (Stafford, 1990). Indirect evidence for this concept comes from

Integrated pathway

↓ *CHS*
chalcone
↓ *CHI*
flavanone
↓ *3-OH*
dihydroflavonol → flavonol
↓ *DHFR*
flavan-3,4-diol → anthocyanidin
↓
flavan-3-ol
↓
proanthocyanidin

Parallel pathways

↓ CHS_1	↓ CHS_2	↓ CHS_3
chalcone	chalcone	chalcone
↓ CHI_1	↓ CHI_2	↓ CHI_3
flavanone	flavanone	flavanone
↓ $3\text{-}OH_1$	↓ $3\text{-}OH_2$	↓ $3\text{-}OH_3$
dihydroflavonol	dihydroflavonol	dihydroflavonol
↓ $DHFR_1$	↓ $DHFR_2$	↓ $DHFR_3$
flavonol	flavan-3,4-diol	flavan-3,4-diol
	↓	↓
	anthocyanidin	flavan-3-ol
		↓
		proanthocyanidin

Figure 5.3: Integrated and parallel pathways to flavonoid end products

demonstration of isozymes (Hille *et al.*, 1982; Ebel, 1986), coordinate increases in enzyme activities and the apparent independent accumulation of major end products (Hahlbrock *et al.*, 1976; Beggs *et al.*, 1987; Brödenfeldt & Mohr, 1988). Direct evidence for parallel pathways has been shown in microscopic investigations with and without immunological tagging (Stafford, 1990).

Various models have been proposed for linear, membrane-oriented, multienzyme complexes (parallel pathways) by Hrazdina & Wagner (1985), Ibrahim *et al.* (1987) and Stafford (1990). The model postulated by Stafford (1990) shows the synthesis of cyanidin-3-glucoside in *Zea mays* (Figure 5.4). Under this system there would be no competition with the proanthocyanidin pathway for the common intermediates dihydroquercetin or the 3,4-diol because the intermediates would be transferred directly, as long as the sequence was not broken due to mutation or other stress. A similar model was also postulated for the synthesis of proanthocyanidins but with a slightly different arrangement of enzymes (Stafford, 1990). As under these systems feedback inhibition would not occur and regulation would be aimed at the

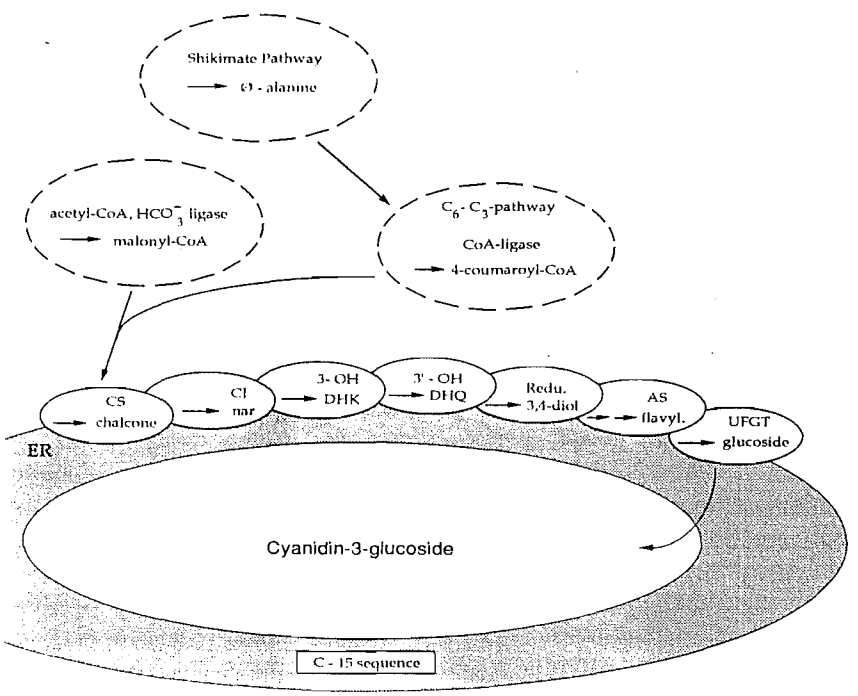


Figure 5.4: Hypothetical model of a membrane-associated C_{15} sequence capable of accumulating cyanidin-3-glucoside. (CS = chalcone synthase; CI = chalcone isomerase; 3-OH = 3-hydroxylase; 3'-OH = 3'-hydroxylase; Redu. = 3hydroxyflavanone hydroxylase; AS = "anthocyanin synthase".) All enzymes are shown facing the cytoplasmic side; the 3'-hydroxylase is firmly bound to the membranes. Three pathways that produce two substrates, malonyl-CoA and 4-coumaroyl-CoA, for the chalcone synthase have been portrayed as three aggregates or vesicles. At the time of synthesis, they could be attached to an adjoining fold of the endoplasmic reticulum to permit direct transfer of the products to the chalcone synthase (from Stafford, 1990).

organization of the complexes.

A similar enzyme sequence to *Z. mays* could be expected for the synthesis of cyanidin-3-galactoside in apples, with a difference in the glycosyltransferase. The position of the 3′-hydroxylase may also be different as in *Z. mays* dihydrokaempferol is known to be the intermediate but in apples this has not been determined. Similar sequences could be expected for the synthesis of flavonols and proanthocyanidins. Independent accumulation of flavonoid end products has been observed in different apple cultivars, with no fixed ratios of division into the various classes (Section Two). Changes occurring in levels of each group during development were similar which would favour the concept of an integrated pathway. However, developmental control mechanisms might influence each of these sequences to flavonoid groups in a similar manner and since slight differences in the patterns of change were observed these may be explained by the parallel pathway concept rather than an integrated system. Measurements of several enzyme activities in apples, to see if there are coordinated increases in activity, may provide further evidence for such a theory of enzyme complexes.

5.2 Developmental regulation of flavonoid enzyme activities

5.2.1 Introduction

5.2.1.1 Phenylalanine ammonia-lyase (PAL)

The reaction catalysed by PAL is the deamination of L-phenylalanine to yield *trans*-cinnamic acid and ammonia (Figure 5.5). This is the first committed step for biosynthesis of the phenylpropanoid skeleton in higher plants (Jones, 1984). There are no cofactor requirements and PAL is sensitive to inhibition by its products. In the time since PAL was discovered in barley seedlings by Koukol and Conn in 1961, it has become the most studied enzyme concerned with secondary metabolism in plants (Camm & Towers, 1973 & 1977). Wide variations in levels of PAL activity in plants have been recorded. Not only does the level depend on the genotype, but also the age and developmental stage, the organ and even tissue type of the plant. Activity of this key enzyme is closely related to the physiological or developmental status of a plant and concomitant increases in the levels of PAL and phenolic compounds have been demonstrated in many tissues (Jones, 1984). In many cases this is also coordinated with the appearance of the other enzymes associated with the flavonoid biosynthetic pathway (Camm & Towers, 1977).

The synthesis of anthocyanin in several plant tissues is associated with increased PAL activity (Tan, 1979) and although anthocyanin production is often associated with an increase in PAL activity there are many examples of PAL activity without anthocyanin production (Camm & Towers, 1977; Jones, 1984). This is due to PAL being active in the biosynthesis of a wide range of phenylpropanoid compounds (Figure 5.6) such as substituted cinnamic acids and their CoA-esters, conjugates of these such as chlorogenic acid, coumarin and lignin (Jones, 1984). PAL activity is also affected by a number of factors including light, temperature, growth regulators, inhibitors of RNA and protein synthesis, wounding and by mineral nutrition (Jones, 1984). PAL has been studied in apple fruit by a number of workers (Table 5.3). Aoki *et al.* (1970) demonstrated PAL activity only in the red sections of the peel and concluded that

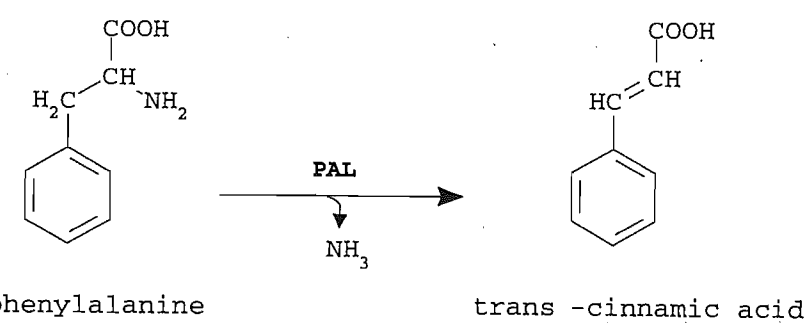


Figure 5.5: Deamination of L-phenylalanine, the reaction catalysed by PAL

PAL activity was closely related to the formation of anthocyanin. Many studies on PAL have used skin disks but results differ markedly between disks and whole skin (Faragher & Chalmers, 1977). The higher levels of PAL activity observed in the disks is probably due to induction due to a wounding response so these PAL levels cannot be directly related to anthocyanin production alone. If there was a direct correlation then a much higher level of anthocyanin synthesis would be expected, compared to the normal tissue, and this has not been observed. Many of the studies have also concentrated on induction of PAL and anthocyanin synthesis by factors such as light. Higher levels of PAL activity are usually observed in these induced tissues (Faragher & Chalmers, 1977). This is probably due to the accumulation of anthocyanins (and possibly other flavonoids) in a period of hours or days, rather than the weeks that they take to accumulate during normal growth.

Table 5.3: Summary of previously reported PAL levels in different apple cultivars (values converted to pKat g^{-1} FW)

Apple cultivar	Tissue type[a]	Treatment	Levels (pKat g^{-1} FW)	Reference
'Starking Delicious'	w	light	8 - 31	Arakawa *et al.* (1986)
"	sd	"	up to 794	
'Fuji'	w	"	15 - 46	
"	sd	"	up to 874	
'Red Delicious'	w	[b]	0 - 0.5	Blankenship & Unrath (1988)
'Golden Delicious'	w	"	0 - 1.2	
'Jonathan'	w	light & temperature	up to 57	Faragher (1983)
'Jonathan'	w	ethylene	14 - 57	Faragher & Brohier (1984)
'Jonathan'	w	light	27 - 124	Faragher & Chalmers (1977)
"	sd	"	413 - 516	
'Fuji'	w	[b]	12 → 0 → 0.5	Kubo *et al.* (1988)
'Jonathan'	w	"	16 → 0 → 2.5	
'Starking Delicious'	w	"	32 → 0 → 8	
'Golden Delicious'	w	"	12 → 0.5 → 3	
'Red Spy'	w	light	7 - 17[c]	Tan (1979)
"	sd	"	8 - 63[c]	

[a] w = skin of whole fruit, sd = skin disks
[b] developmental changes
[c] figure in pKat mg^{-1} protein

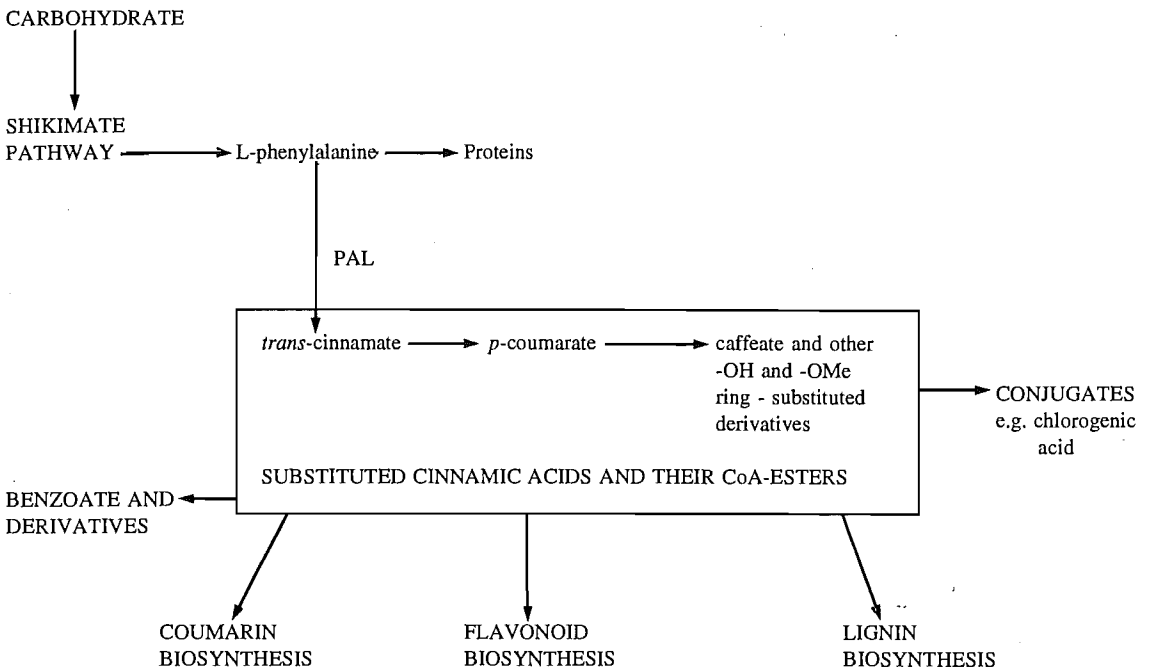


Figure 5.6: General outline of phenylpropanoid metabolism (adapted from Jones, 1984)

Although there have been many studies on the effect of light on PAL activity (Faragher & Chalmers, 1977; Tan, 1979; Faragher, 1983; Arakawa *et al.*, 1986) there has been little study of the developmental changes in PAL activity in apples (Blankenship & Unrath, 1988; Kubo *et al.*, 1988). There was a wide variation in the levels of PAL activity between cultivars but in all cultivars PAL activity was highest in the immature fruit, dropping to very low levels during growth. This was followed by a rise on ripening, coinciding with an increase in anthocyanin concentration in red cultivars but fruit, such as 'Golden Delicious', that did not accumulate anthocyanins still showed a rise in PAL activity (Kubo *et al.*, 1988). However, these studies have related PAL activity to anthocyanin content only and not total flavonoids. The increase in PAL activity, observed in ripening in 'Golden Delicious' by Blankenship & Unrath (1988), may be related to an increased synthesis of flavonoid classes other than anthocyanins. PAL has been studied in apple cell cultures and activity changes showed a typical parabolic curve with a maximum after three days of culture growth and then a sharp decline. However, the flavonoids continued to accumulate after the decline in PAL activity (Macheix *et al.*, 1981).

The recent finding that a PAL inactivating system (PAL-IS) was capable of inactivating PAL *in vitro* led to increased study of its possible role as a regulator of PAL in the plant (Tan, 1979). PAL-IS has been demonstrated in a number of plant extracts including leaf disks of sunflower (Creasy, 1976), sweet potato root (Tanaka *et al.*, 1977) and also apple skin and leaves (Tan, 1979). Also a macromolecule inhibitor of PAL has been reported in gherkin hypocotyls (French & Smith, 1975). Tan (1980) studied the relationship between PAL and PAL-IS in apple and concluded that low temperature reduced the level

of PAL-IS which resulted in increased accumulation of PAL which, in turn, increased anthocyanin accumulation in the skin of whole apples. Nitrogen and potassium deficiencies also reduced the level of PAL-IS and therefore increased the accumulation of PAL in apple leaves.

The main aims of the present study were to follow changes in PAL during the growth and ripening in red ('Splendour') and green-skinned ('Granny Smith') New Zealand-grown apple cultivars. By comparison of a red and a green cultivar some of the enzyme changes related to reddening could be compared to see where these differed from changes taking place on ripening only. The two cultivars studied have been shown to have different rates of flavonoid synthesis (Section Four). Variations in the levels of enzyme activities were determined to see if these accounted for these differences. Other factors involved in controlling synthesis rates, such as the levels of inhibitors, were investigated. PAL activity was also measured for a range of apple cultivars with differing anthocyanin concentrations to see if enzyme activity related to pigment accumulation.

5.2.1.2 Chalcone isomerase (CHI)

It was only established recently that the isomerase step was a part of all major flavonoid pathways (Stafford, 1990). The first intermediate in the flavonoid pathway at the C_{15} level is now believed to be an open chain flavonoid, a chalcone, formed by chalcone synthase. Since the monophenol, 4-coumaroyl-CoA, is the usual substrate for the first enzyme, the first intermediate for all flavonoids has a monohydroxy B-ring as in naringenin chalcone (2′,4,4′,6′-tetrahydroxychalcone). Early ^{14}C feeding studies with seedlings were interpreted as indicating the chalcone as the initial precursor of other flavonoids (Wong, 1968). However, until recently naringenin, the isomeric flavanone with a central heterocyclic ring, was believed by many to be the first intermediate because it accumulated in incubation mixtures, with cell-free extracts, as the first stable intermediate (Stafford, 1990). The first enzyme in the flavonoid pathway was therefore called "flavanone synthase" (Kreuzaler & Hahlbrock, 1975). Evidence from cell-free extracts now indicates that the chalcone is the first condensation product in a two step process (Heller & Hahlbrock, 1980). The term "flavanone synthase" might still be a useful name if the first two enzymes are always found together as a tight complex during active synthesis of the flavanone (Figure 5.7). Such a complex would be a useful device to prevent the non-enzymic formation of a flavanone with the 'wrong' stereochemistry at C-2 (*R* rather than *S*). If the two enzymes were not closely associated non-enzymic isomerisation of the chalcones could occur (Stafford, 1990). Since all known reactions of flavonoid biosynthesis originate from (2*S*)-flavanone, the importance of the isomerase is evident and has been confirmed by studies with defined genotypes from several plants (Beggs *et al.*, 1986). Both synthase and isomerase activity are induced at the same time under certain conditions, and they generally tend to change in a similar pattern during development (Heller & Hahlbrock, 1980; Stafford, 1990).

Chalcone isomerase was first isolated and purified from soya bean seed (*Soja hispida*) by Moustafa & Wong (1967). Activity of the enzyme can be assayed by following the decrease in absorbance at 360-

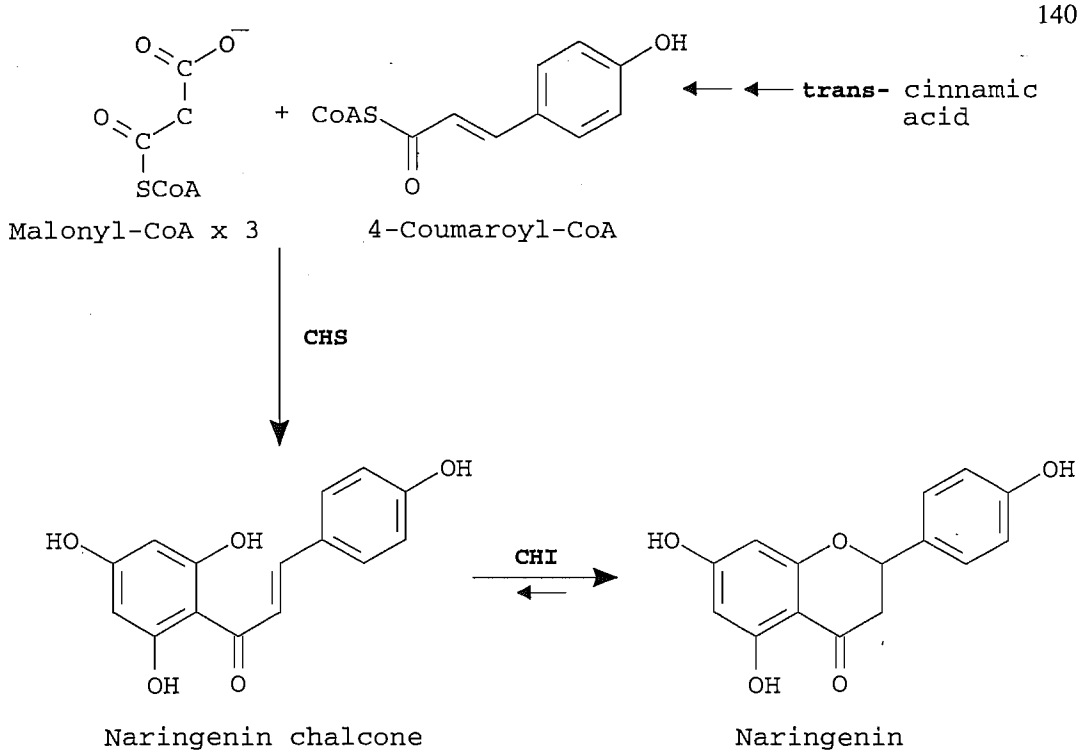


Figure 5.7: Synthesis of naringenin via chalcone synthase (CHS) and chalcone isomerase (CHI)

380 nm due to the chalcone disappearance but non-enzymic cyclisation and peroxidatic activity are major problems. Peroxidase can catalyse extremely rapid oxidation of chalcones (Wilson & Wong, 1976) which would otherwise interfere with isomerase measurement (Boland & Wong, 1976; Dixon *et al.*, 1982) but high concentrations of KCN can be added to inhibit these. Studies on *Petunia* isomerase, by Mol *et al.* (1985), showed that the presence of high concentrations of albumin severely depressed the self-cyclisation of the chalcone at pH values between 5.5 and 9.0. Under these conditions the change in absorbance can be assigned to stereospecific chalcone isomerisation and thus measurement of enzyme activity alone. Thus, these precautions were taken when measuring CHI activity in apple fruit development in the two cultivars as for PAL activity.

5.2.1.3 Flavonoid glycosyltransferase (GT)

Anthocyanins are rarely found as aglycones since glycosylation at the 3-carbon position occurs immediately (Figure 5.8) because the 5,7-hydroxy-flavylium nucleus and its structural variants are unstable (Stafford, 1990). The first stable products are 3-*O*-glycosides with glucose generally being the most common substituent. UDP-3-*O*-glucosyltransferases (UFGT) have been demonstrated for various anthocyanins in seedlings, flower petals, and seed coats. These enzymes are able to glycosylate more than one type of anthocyanidin B-ring and some can also glycosylate flavonols, but generally not dihydroflavonols (Stafford, 1990). No aglycones of the flavonols, such as quercetin, were seen in apples

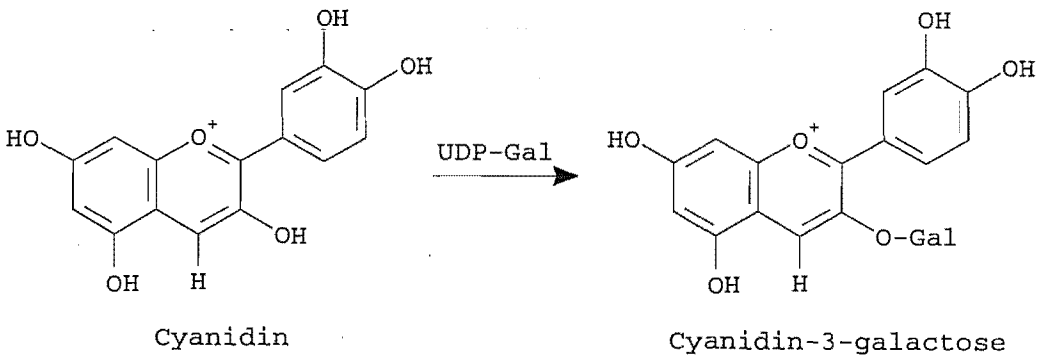


Figure 5.8: Glycosylation reaction of the flavonoids catalysed by UDP-flavonoid glycosyltransferase (GT)

and all flavonols were present as glycosides and for both the flavonols and the anthocyanins the galactoside was generally the predominant sugar. This suggests that either there is one set of glycosyltransferases able to glycosylate both the anthocyanidins and the flavonols or there are two sets with similar sugar specificities. There are a multitude of well-known glycosyltransferases and a large number have been characterized in recent years (Heller & Forkmann, 1988). Novel flavonol glycosyltransferase enzymes have been reported from *Tulipa* anthers (Kleinehollenhorst *et al.*, 1982), *Pisum* flowers (Jourdan & Mansell, 1982), *Chrysosplenium* shoots (Bajaj *et al.*, 1983) and *Anethum* cell cultures (Möhle *et al.*, 1985). These enzymes exhibit pronounced specificity with regard to the substrate, the position and the sugar transferred (Stafford, 1990).

An extensive study of glycosylation in *Silene pratensis* and *S. dioica* has identified ten different glycosyltransferases which control glycosylation of the flavone isovitexin (Steyns *et al.*, 1984). Isovitexin may be glycosylated at the 7-OH of the A-ring by either galactose, glucose or xylose and at the 2"-OH of the C-C bound glucose by either glucose, xylose, rhamnose or arabinose to give the disaccharide (van Brederode *et al.*, 1987). Each enzyme catalyses a different glycosylation reaction such as glucosylation, galactosylation or xylosylation at a different position. The 7-O-glucosyltransferase can distinguish between glucose and its stereoisomer galactose. These enzymes act in the petals and/or different developmental stages of the green parts. Serological studies have shown that the various isovitexin glycosyltransferases are evolutionarily related (van Brederode *et al.*, 1987). A similarly large number of glycosyltransferases could be expected to be found in apples due to the range of flavonol glycosides present.

The only study of glycosyltransferases in apples is one on glucosyltransferases and phenolic compounds in apple cell suspension culture by Macheix *et al.* (1981). It was shown that GT activity exhibited a gradual, but constant, increase during culture growth which took place in parallel with the accumulation of phenolic compounds. This was more prominent with kaempferol than with quercetin and they concluded that glycosylation was a terminal step in the biosynthesis of phenolic compounds. This

activity pattern compared well with that of soybean cell culture but was different from that of rose and tobacco cell cultures. In their study glucosyltransferase was examined but the main sugar attached to the apple flavonoids is galactose, which was not studied. It is also interesting that Macheix *et al.* (1981) found higher glucosyltransferase activity for kaempferol than quercetin, because in apples quercetin is the natural substrate and kaempferol is present only at low levels. Thus, lack of glycosylation is not an explanation for the absence of kaempferol glycosides. The substrate specificity of the glycosyltransferases, with relation to the sugar donors, was examined to see if this corresponded with the ratios of quercetin glycosides observed in the fruit. Unfortunately only a limited number of UDP sugars are available commercially, being UDP-galactose, -glucose and -xylose. Unfortunately arabinose and rhamnose, which are present in the flavonol glycosides of apples, were not available in UDP form and thus could not be tested. Substrate specificity with regard to the chemical structure of the aglycone was also examined. As with PAL and CHI the changes in GT activity were measured during the development of two apple cultivars.

5.2.1.4 Extraction of enzymes

There are many problems that can be experienced with the extraction of enzymes from plant tissues and these have been comprehensively reviewed (Loomis & Battaile, 1966; Anderson, 1968; Rhodes, 1977; Gegenheimer, 1990). In apples there are several specific problems in the extraction of active enzymes since apples contain very low levels of proteins compared to many other tissues at approximately 0.2% of fresh weight (Rhodes, 1977). The activity of the flavonoid enzymes is also likely to be low since they are synthesised over a longer period than other tissues, such as flowers. For these reasons large amounts of tissue needed to be sampled, however this was beneficial in that it eliminated any variation that may be due to distribution of activity in different parts of the apple, such as sun and shade sides of the fruit. Because a large sample was needed, the fruit had to be allowed to develop for a few weeks before sampling could be started.

Large concentrations of phenolics are also present in the apple which can inhibit enzyme activity (Loomis & Battaile, 1966). These can be removed from the crude enzyme extract by complexing with agents such as PVP or PVPP. In apples it was shown that only soluble PVP gave protection for NADP-malic enzyme and insoluble PVPP gave about 70% activity (Rhodes, 1977). The use of borate buffers and sodium metabisulfite can also be beneficial (Gegenheimer, 1990). It was also necessary to protect enzymes from the products of enzymic browning, which could be achieved by the use of soluble reducing agents and phenolase inhibitors (Anderson, 1968). A number of compounds can be used including mercaptoethanol, dithiothreitol (DTT), and ascorbate.

Large variations in PAL activities have been reported and whether these variations were due to actual differences in activity or variation arising from the different methods used is unclear. The methods used have been similar, with some variation in the protective agents added to the buffer. Borate buffer

has been used with the addition of PVPP (Tan, 1979), the addition of mercaptoethanol (Faragher & Chalmers, 1977) and EDTA (Blankenship & Unrath, 1988) have also been reported. This was be used as a starting point to develop a method for the extraction of the flavonoid enzymes from apple tissue. It would be beneficial to be able to measure all enzyme activities from a single extract, to provide direct comparison of enzyme activities which could be related to flavonoid concentrations. The extraction of the glycosyltransferase(s) required the addition of Triton X-100 for solubilisation of the enzyme (Heinsbroek *et al.*, 1979) and it was necessary to check that this didn't interfere with the activity of the other enzymes measured.

5.2.2 Materials and methods

Materials

Buffers

Buffer A: 50 mM phosphate buffer (Na_2HPO_4/KH_2PO_4) pH 7.0 containing 5% PVP (MW 44,000), 50 mM Na ascorbate, 18 mM mercaptoethanol and 0.1% Triton X-100.

Buffer B: as for buffer A but without PVP and Triton.

Buffer C: 0.1 M MES buffer at pH 5.5.

Chemicals

L-Phenylalanine (L-Phe), trifluoroacetic acid (TFA), cinnamic acid, NADPH, UDP-galactose, UDP-glucose and UDP-xylose were obtained from Sigma Chemical Co. (U.S.A.).

Glucose-6-phosphate and glucose-6-phosphate dehydrogenase were obtained from Boehringer Mannheim (P.O. Box 310120, 6800 Mannheim 31, Germany).

Flavonoid standards

Naringenin, kaempferol, myricetin, quercetin, taxifolin ((+)-dihydroquercetin), UDP-galactose, UDP-glucose, UDP-xylose and vanillin were obtained from Sigma Chemical Co. (U.S.A.).

Cyanidin chloride, delphinidin chloride, eriodictyol, fisetin, isorhamnetin, luteolin, malvidin chloride, pelargonidin chloride and rhamnetin were obtained from Extrasynthese (Z.I. Lyon Nord, B.P. 62, 69730 Genay, France).

Plant material

Apples were obtained from the Lincoln University Orchard.

Preparation of flavan-3,4-diol (leucocyanidin)

The flavan-3,4-diol, for the reductase assay, was synthesized by non-enzymic conversion of dihydroquercetin using the method of Stafford & Lester (1982). Five mg (+)-dihydroquercetin was stirred into 1 ml of ethanol and 3 mg $NaBH_4$ was added over a 30 min period. After one hour 2 ml 0.1 M HCl and 10 ml of H_2O was added and the mixture extracted immediately with 6 x 1 ml of ethylacetate; the latter was backwashed with 3 x 0.2 ml H_2O. The ethylacetate was evaporated under vacuum, at 40°C, down to 0.5 ml and 0.5 ml of H_20 added then the remaining ethylacetate evaporated. The aqueous extract was added to a C_{18}-mini column (Sep-Pak) and eluted with MeOH:H_2O (70:30). This fraction, containing the 3,4-diol, was collected and analysed by HPLC.

Synthesis of tetrahydroxychalcone

2′,4,4′,6′-Tetrahydroxychalcone (naringenin chalcone) was synthesized from naringenin using the method of Moustafa and Wong (1967), by treatment with 50% KOH followed by acidification and recrystallization from aqueous ethanol.

Methods

Handling of samples

Apples were harvested and the skin removed carefully taking care to remove all underlying flesh. The skins of individual apples were combined to give samples of about 20 g. Samples were frozen in liquid nitrogen and if not used immediately were stored at -80°C.

Extraction of enzyme activity

Skin (usually 20 g) was ground to a fine powder with a mortar and pestle using liquid N_2. The powder was then extracted in buffer A (100 ml), the homogenate filtered and centrifuged at 20,000 g for 10 min. Ammonium sulphate was added to the supernatant to give 35% saturation and centrifuged for 20 min at 20,000 g to remove the PVP. Further ammonium sulphate was added to this supernatant to reach a final saturation of 80%. This fraction was centrifuged at 20,000 g for 20 min and the pellet resuspended in a small volume of buffer B (5-10 ml). This solution was then dialysed overnight against buffer B to give a crude extract that was used for all enzyme assays. All these procedures were carried out at 4°C.

Estimation of protein concentration

Protein was estimated by the method of Bradford (1976) using BSA as a standard. Samples were usually diluted 10 to 20-fold with 50 mM phosphate buffer to give readings in the linear range of the standard curve.

Assay of PAL activity

PAL activity in the crude enzyme extracts was assayed by an adaptation of the method of Zucker (1965) as reported by McCallum & Walker (1990a). Assays were performed in Eppendorf tubes. The assay mixture consisted of 0.06 M borate buffer (875 µl) and crude enzyme (250 µl). The reaction was initiated by the addition of L-Phe (250 µl of 10 mg/ml to give a final concentration of 11 mM). Tubes were incubated at 30°C for 30 min, or one hour when activity was low, and the reaction stopped by addition of 35% w/v TFA (125 µl). Tubes were then centrifuged for 5 min at 5000 g to pellet the denatured protein. The yield of cinnamic acid was estimated by measuring A_{290} of the supernatant in 1 cm quartz cuvettes. Triplicate assays were performed for each extract, both with and without substrate in order to compensate for increases in absorbance even in the absence of added L-Phe.

Analysis of PAL activity in different cultivars

A range of different coloured apple cultivars were sampled at three times during the growing season (early, middle and late). The skins of these fruits were prepared, extracted and assayed for PAL activity by the methods outlined above. Three samples were taken at each collection point. For comparison activity was also measured in apple flower buds, crab apples and red pear skin. Anthocyanin and total flavonoids were also measured for each of the samples as outlined below.

Extraction and assay of PAL-IS

Extraction and assay of PAL-IS in apple skin was carried out by the method of Tan (1979). Skin samples were ground to a fine powder with liquid N_2 and 100 mg PVPP added. Buffer C was added and the homogenate centrifuged at 2,000 g for 5 min. The pellet was discarded and the supernatant centrifuged at 20,000 g for 15 min. Sodium borate was added to the supernatant until the pH reached 8.8 and then centrifuged at 1,000 g for 10 min to remove excess Na borate. This final supernatant, containing no PAL activity, was used immediately for assay of PAL-IS.

PAL-IS was assayed by adding a known amount of PAL (from a Camellia flower extract) to 50 µl of the supernatant plus 40 µl of 0.1 M Na borate buffer pH 8.8. The tubes were incubated at 30°C and samples taken at time 0 and 15 min. The control consisted of PAL extract from Camellia flower and buffer only.

Assay of CHI activity

Crude enzyme (50-200 µl) was added to 50 mM Tris-HCl pH 7.4 containing BSA (at a final concentration of 7.5 mg/ml) and 50 mM KCN to give a final volume of 1 ml. The reaction was initiated by adding 5 µl of 1 mg/ml tetrahydroxychalcone in 2-ethoxyethanol (final concentration 18.5 µM). Absorbance at 381 nm was measured in a dual beam spectrophotometer with the cell holder maintained at 30°C. The reference cell contained the assay mixture without enzyme, to account for spontaneous isomerisation of the chalcone. The initial rate of disappearance of the chalcone, in the presence of

enzyme, was used to estimate CHI activity. All samples were assayed in triplicate.

Assay of GT activity

Assay mixtures contained crude enzyme (100 µl), 50 mM bicine buffer pH 8.5 (100 µl), substrate (quercetin - 15 µl of 2 mg ml^{-1}, final concentration 1 mM) and UDP-galactose (10 µl of 15 mg ml^{-1}, final concentration 2.5 mM). Tubes were incubated at 30°C for 10 to 30 min and the reaction stopped by the addition of 20% TCA in methanol (75 µl). Tubes were then centrifuged for 5 min at 5000 g and the supernatant was then stored at -20°C, before quantification by HPLC.

Reaction products were quantified by HPLC using an adaptation of the usual HPLC method (Section Two) but because there was only a single compound in each sample it was possible to use isocratic elution, which shortened the run time. Solvents used were: (A) aqueous acetic acid (10% v/v) and (B) acetonitrile. The solvent was pumped isocratically using a solvent mixture of A:B = 85:15, and at a flow rate of 1 ml min^{-1}. Eluted components were detected at 350 nm and data acquisition time was 10 min, with quercetin-3-galactoside eluting at 5.0 min.

Substrate and sugar specificity of GT

A range of substrates (Table 5.4) was assayed for glycosyltransferase activity using the assay method outlined above. All substrates were assayed with UDP-glucose and UDP-galactose as the sugar donors and some were also assayed with UDP-xylose. An enzyme preparation from 'Splendour' apple skin was used for all assays. All activities were related to that with quercetin as substrate and UDP-galactose as the sugar donor (taken as 100% activity). HPLC analysis was performed as outlined above with the anthocyanins being monitored at 530 nm, dihydroquercetin and eriodictyol at 280 nm and all others at 350 nm. Where standards were not available, quantification was based on cyanidin-3-galactoside for the anthocyanins, and quercetin-3-galactoside for the others. Although this gave only approximations similar response factors were observed within a given class of compounds.

Reductase assay

The two reductases (dihydroflavonol and diol) were assayed in a double reaction, by adaptation of the methods of Stafford and Lester (1982, 1984 & 1985). A 1 ml reaction mixture contained 50 mM Tris buffer pH 7.5, 1 mM NADPH, 6 mM glucose-6-phosphate, 1 unit glucose-6-phosphate dehydrogenase, 1 mM substrate (dihydroquercetin or 3,4-diol) and crude enzyme extract (usually 250 µl). After incubation for one to three hours the mixture was extracted with 2 x 0.2 ml ethylacetate. The products contained in the ethylacetate fraction were estimated by the following methods:

(1) Vanillin estimation for catechins and leucoanthocyanidins (Broadhurst & Jones, 1978): The reaction mixture consisted of 0.1 ml sample (ethyl acetate fraction), 0.6 ml vanillin solution (4% w/v vanillin in methanol) and 0.3 ml conc. HCl. The mixture was incubated at 20°C for 15 min, in the dark, and the absorbance read at 500 nm.

Table 5.4: Structures of flavonoid aglycones used for glycosyltransferase substrate specificity experiments

Flavonoid aglycone	Structure
Anthocyanidins	
Cyanidin	3,5,7,3′,4′-OH
Delphinidin	3,5,7,3′,4′,5′-OH
Malvidin	3′,5′-Me delphinidin
Pelargonidin	3,5,7,4′-OH
Dihydroflavonol	
Taxifolin	3,5,7,3′,4′-OH
Flavanone	
Eriodictyol	5,7,3′,4′-OH
Flavone	
Luteolin	5,7,3′,4′-OH
Flavonols	
Fisetin	3,7,3′,4′-OH
Isorhamnetin	3′-Me quercetin
Kaempferol	3,5,7,4′-OH
Myricetin	3,5,7,3′,4′,5′-OH
Quercetin	3,5,7,3′,4′-OH
Rhamnetin	7-Me quercetin

(2) Butanol-HCl reagent for leucoanthocyanidins (Stafford & Lester, 1980): Aliquots of 0.1 ml sample were heated with 1 ml butanol-HCl (95:5, v/v) at 95°C for 30 min. The absorbance was determined at 550 nm, the λ_{max} for cyanidin.

Assay of flavonoid 3′-hydroxylase

The standard crude enzyme extract could not be used for this assay since the enzyme was microsomal. Microsomal fractions were prepared by Mg^{2+} precipitation of extracts by several different methods (Forkmann *et al.*, 1980; Britsch *et al.*, 1981; Hagmann *et al.*, 1983).

The assay consisted of 0.1 M potassium phosphate buffer (pH 7.5), 0.5 mM NADPH, 2 mM mercaptoethanol, 0.35 mM kaempferol and enzyme (10-50 µg protein). The reaction mixture was mixed thoroughly and incubated, in open Eppendorf tubes, for 30 min at 30°C in a shaking waterbath. The reaction was stopped by the addition of 2.5 mg EDTA and extracted with 2 x 50 µl ethylacetate. This

ethylacetate fraction was used for flavonol estimation by addition of 50 µl sample to 1 ml of Naturstoffreagenz A (2% w/v diphenyl-boric acid-ethanolamine complex in methanol). The absorbance of the solution was then read over the range 300 to 600 nm.

Determination of K_m values

The K_m values were measured using the standard assays as outlined above. Lineweaver-Burk plots were used for the determination of the K_m values.

Changes in enzyme activity during fruit development

Samples were taken of 'Granny Smith' and 'Splendour' at weekly or fortnightly intervals over the 1992/93 apple season. Sampling commenced in mid-December and ended in late April at normal harvest time, with a total of 14 samples. Samples were taken at the same time of day (early morning) at each sampling point and handled in the same way. Extractions and assays were performed as detailed above and performed in triplicate for each sampling time.

Quantification of flavonoids

A small sample (0.5 g) was taken from each enzyme sample and set aside for flavonoid quantification by HPLC. The method used was improved slightly and all flavonoid groups (i.e. anthocyanins, flavonols and proanthocyanidins) were quantified from a single chromatogram. There were two differences from the method given in Section Two: (1) the column was maintained at 30°C; (2) a linear 20 min solvent gradient from 0 to 20% acetonitrile, with a 10 min hold at the final concentration was used. Eluted components were monitored at 280 nm for proanthocyanidins, 313 nm for phenolic acids, 350 nm for flavonols and 530 nm for anthocyanins. The individual compounds were identified and quantified by comparison with standard solutions of known concentration.

Statistical analysis

Enzyme activity was expressed in pKat per gram fresh weight so that activity could be directly compared to changes in total flavonoid and anthocyanin concentrations. Calculations were also made of total amount of enzyme activity per apple. Surface area of the fruit was calculated from fruit diameter assuming a spherical shape. Standard ANOVA established significant differences among cultivars over time, so subsequently each cultivar was analysed separately. All LSD's are given at the 5% significance level.

Concentrations and total amounts of anthocyanins and flavonoids were measured and calculated for each fruit. Regression analyses on enzyme activities and flavonoid concentrations were carried out using the spreadsheet program Quattro Pro.

5.2.3 Results

Determination of K_m values

The K_m value for PAL from 'Splendour' apple skin was 0.14 mM and for 'Granny Smith' the K_m was 0.19 mM. The K_m for CHI from 'Splendour' apple fruit skin was approximately 91.4 μM and for 'Granny Smith' the K_m was 93.9 μM. These values for CHI may be slightly overestimated since the tetrahydroxychalcone concentration could only be calculated approximately as it was not 100% pure.

Analysis of PAL activity in different cultivars

There was a wide variation in the level of PAL activity in different apple cultivars (Table 5.5) and the values shown may not reflect the full range of activity seen in each cultivar as, in most cases, the figures are based on samples taken at only three stages of fruit development (early, middle and late). There was no correlation between average PAL activity and final anthocyanin concentration ($r=0.34$, $p>0.05$), but there was a significant correlation between average PAL activity and final concentration of total flavonoids ($r=0.73$, $p<0.02$).

In 'Red Delicious' the PAL activity did not drop as low as many other cultivars such as 'Splendour'. Measurements of activity at several points during development of 'Red Delicious' showed that activity was more constant throughout development and did not show the large drop in the middle of the season shown by other cultivars such as 'Granny Smith' and 'Splendour'. Similarly, the pattern of anthocyanin accumulation, based on visual observation, was different in 'Red Delicious' compared to 'Splendour'. 'Red Delicious' showed a more gradual accumulation of anthocyanin over the whole season rather than the dramatic rise in the last month of development as did 'Splendour'.

PAL activity in flowers, particularly in the bud stage, was much higher than the fruit although the anthocyanin concentration was not particularly high, with the flowers being only pale pink. However, the concentration of total flavonoids in the flower was higher than the fruit. PAL activity in the skin of the crab apple and red pear samples was similar to apple skin. These tissues also had similar flavonoid concentrations.

PAL-IS

The differences in PAL-IS between the cultivars 'Granny Smith' and 'Splendour' were insignificant, both having similar levels of PAL-IS at all three stages. From initial investigations at four points during the season, there was a small drop in PAL-IS level from early to middle stages of fruit development and then it remained relatively constant with no significant changes in PAL-IS during ripening when the anthocyanins increased. Thus, it was not measured throughout the season in detail as for PAL and the other enzymes.

Table 5.5: PAL activity in different apple cultivars and other tissues

Sample type	Flavonoid levels (mg g^{-1} FW)[a]		PAL levels (pKat g^{-1} FW)	
	Anthocyanin	Total	Min.	Max.
Apple Cultivar				
'Cox's Orange'	0.32	8.27	1.78	21.20
'Fuji'	0.09	10.56	2.36	31.89
'Gala' cross	0.49	7.46	0.88	26.00
'Golden Delicious'	0.00	3.14	0.00	8.01
'Granny Smith'	0.00	5.69	0.00	10.26
'Ikorokavka Alajah'[b]	0.52	7.58	-	85.72
'Jonagold'	0.37	5.42	0.90	9.39
'Red Delicious'	2.59	8.92	4.21	11.47
'Royal Gala'	2.31	8.14	0.92	18.32
'Splendour'	0.97	9.56	2.20	38.57
Other Tissues				
Apple Flower (pink)	0.11	12.34	20.32[c]	275.40[d]
Crab Apple[b]	0.75	7.21	-	13.85
Red Pear[b]	0.23	5.69	-	8.82

[a] Concentrations in fruit close to maturity (late sampling)
[b] Only one sampling point - fruit close to maturity
[c] Fully opened flowers
[d] Buds (unopened)

Substrate and sugar specificity of glycosyltransferase

A crude enzyme preparation from 'Splendour' apple skins glycosylated a range of flavonoid substrates with different sugars to varying degrees (Table 5.6). Only one compound was observed from each reaction and, based on retention time and comparison to standards where possible, all compounds were glycosylated at the 3-position. Of the three sugars galactose showed the highest activity with all substrates followed by glucose and then xylose, although xylose was tested with only a few substrates. There were only three substrates that showed no activity with galactose or glucose, these were eriodictyol, luteolin and taxifolin (dihydroquercetin) but none of these compounds are usually observed in apples (except for small traces of taxifolin, which was not glycosylated). The flavonols kaempferol (4'-OH),

Table 5.6: Substrate specificity of the *O*-glycosyltransferases from apple skin

Substrate	Relative activity (%)[a]		
	galactose	glucose	xylose
Cyanidin	217.1	14.7	1.1
Delphinidin	92.4	5.1	-
Eriodictyol	n.d.	n.d.	-
Fisetin	41.3	5.6	-
Isorhamnetin	93.2	4.3	-
Kaempferol	114.3	5.9	3.1
Luteolin	n.d.	n.d.	-
Malvidin	1.4	0.8	-
Myricetin	124.3	11.0	0.73
Pelargonidin	68.7	1.5	-
Quercetin	100	31.2	25.7
Rhamnetin	2.6	2.1	-
Taxifolin	n.d.	n.d.	-

[a] Relative activity is given as a percentage of the activity determined with quercetin and galactose as substrates, which was taken as 100%.
- Assay not performed
n.d. No activity detected

quercetin (3′,4′-OH) and myricetin (3′,4′,5′-OH) all showed similar levels of activity. Cyanidin showed the highest level of activity, of all the flavonoids tested, with galactose but with glucose the activity was lower than for quercetin. Of the methylated flavonoids isorhamnetin showed significant activity but rhamnetin and malvidin showed very low activity.

Changes in enzyme activity during fruit development

Calculation of enzyme activity in terms of pKat mg^{-1} protein did not significantly alter the patterns of change of the enzymes. On average the protein levels in the final preparations were slightly higher in 'Splendour' (0.27 mg g^{-1} FW) than 'Granny Smith' (0.23 mg g^{-1} FW). However, the changes in protein concentration during development were not significantly different between the two cultivars. Thus, all results are discussed in terms of pKat per g FW to allow direct comparison with anthocyanin and total flavonoid concentrations.

(a) PAL

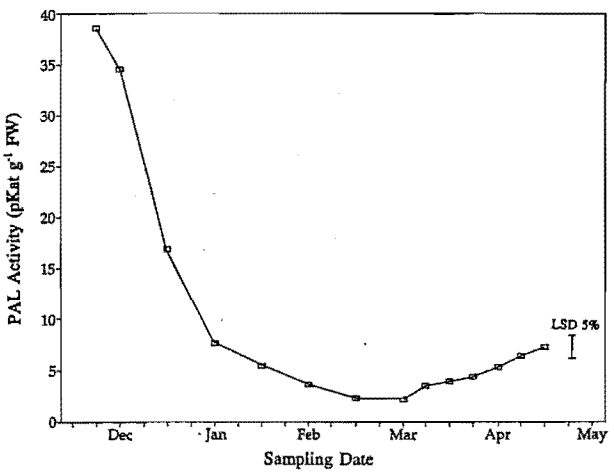
The same pattern of change in PAL activity was observed in the skin of both apple cultivars, 'Splendour' (Figure 5.9 a) and 'Granny Smith' (Figure 5.10 a), although the level of activity in 'Splendour' was approximately four times that of 'Granny Smith', at all stages of development. In both cultivars PAL activity was highest at first sampling in mid-December, 'Splendour' at 38.6 pKat g^{-1} FW and 'Granny Smith' at 11.3 pKat g^{-1} FW. The activity dropped rapidly over the next five weeks, then the decline became more gradual through to early March. At this point the activity in 'Splendour' was only 6% of the initial measurement (2.2 pKat g^{-1} FW) and in 'Granny Smith' no activity was detected. Even though no activity was detected at this point it was likely that there was still a low level of activity, however in this preparation it was below the detection limits of the assay system. From mid-March until the final harvest, at the end of April, there was a steady rise in PAL activity in both cultivars. The final levels reached were only about 20% of the original; in 'Splendour' this was 7.3 pKat g^{-1} FW and in 'Granny Smith' 3.2 pKat g^{-1} FW. The observed decrease in PAL activity did not appear to be caused by inhibition of PAL since activities were additive when extracts from immature fruit were mixed with those from the middle stages of development or mature fruit.

When PAL activity was calculated in terms of total activity per apple, the pattern of change was similar in both cultivars (Figures 5.11 a & 5.12 a) with a drop from high initial levels of 58.2 pKat per apple in 'Splendour' and 16.3 pKat per apple in 'Granny Smith'. This decrease in PAL activity continued through to early March and was followed by a steady rise. There were several small differences, compared to the pattern on a concentration basis, the first was that there was a small rise in activity between the first and second sampling which was more prominent in 'Splendour'. The rise was 15% for 'Splendour' and for 'Granny Smith' it was smaller, with a 4.5% increase only. A difference was also noted in the relative size of the drop in activity to the middle of the season. In early March about 25% of the original level of activity remained in 'Splendour' although none was detected for 'Granny Smith'. A final difference was in the final level of activity reached, with a more pronounced rise in the amount of activity in 'Splendour' through to the end of the season. Final levels of PAL activity were similar to the initial levels at 64.6 pKat per apple for 'Splendour' and 21.0 pKat per apple for 'Granny Smith'.

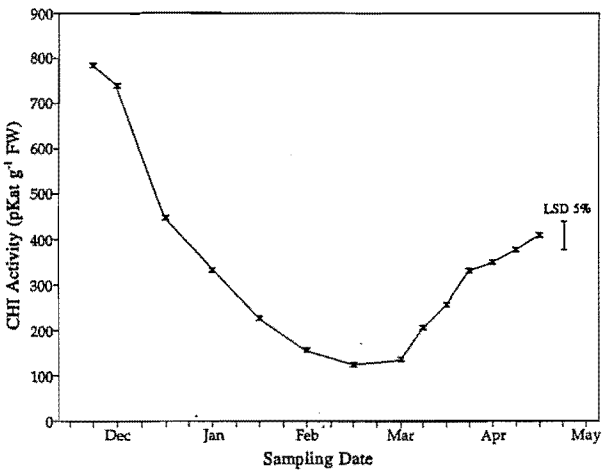
(b) CHI

The pattern of change in CHI activity was similar to that observed for PAL although the level of CHI activity was significantly higher than PAL, by about 20-fold for 'Splendour' (Figure 5.9 b) and 30-fold for 'Granny Smith' (Figure 5.10 b). As with PAL the level of CHI activity was two to four times higher for 'Splendour' compared to 'Granny Smith', at all stages of growth. Initial levels of CHI activity, in mid-December, were 785 pKat g^{-1} FW in 'Splendour' and 345 pKat g^{-1} FW in 'Granny Smith'. In both cultivars these levels dropped through to early March when 'Splendour' CHI activity was at 16% of the original level (126 pKat g^{-1} FW). In 'Granny Smith' no PAL activity was detected in mid-development but there was still some CHI activity, although this was only 8% of the original activity (28 pKat g^{-1} FW).

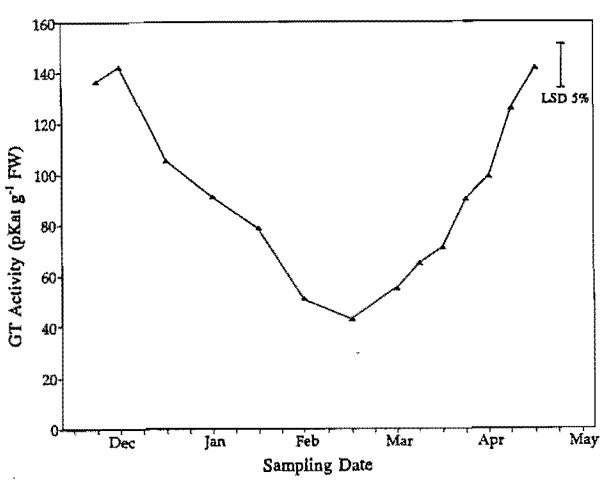
(a) PAL



(b) CHI



(c) GT



(d) Flavonoid Concentrations

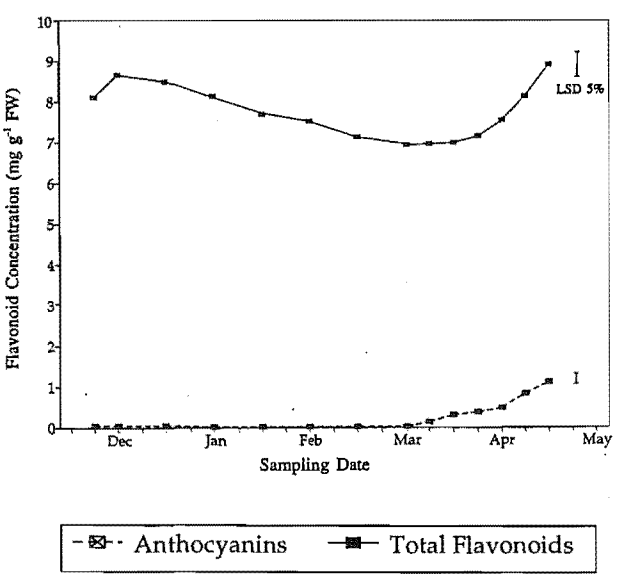
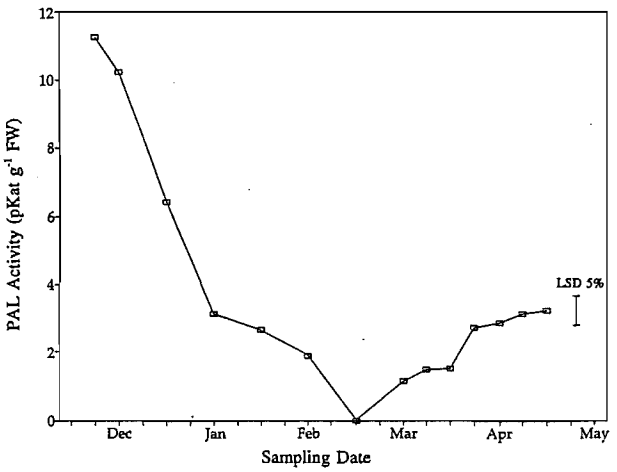
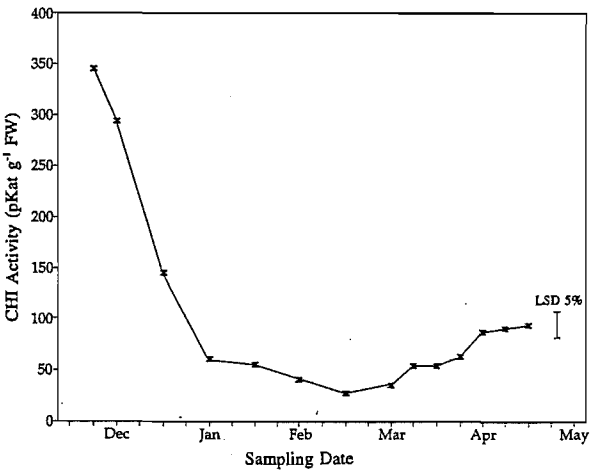


Figure 5.9: Changes in enzyme activities and flavonoid concentrations in 'Splendour' apples during development

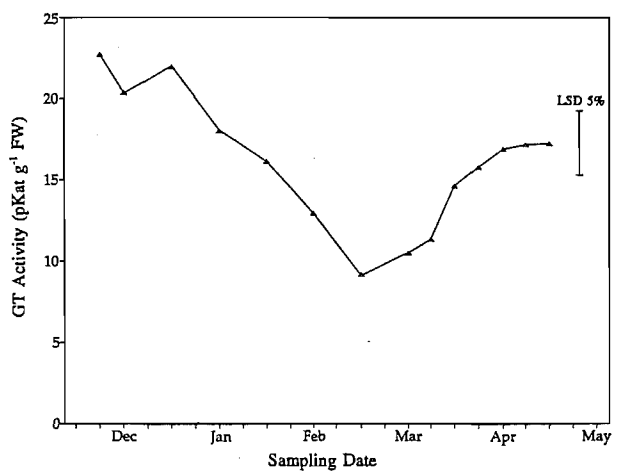
(a) PAL



(b) CHI



(c) GT



(d) Flavonoid Concentrations

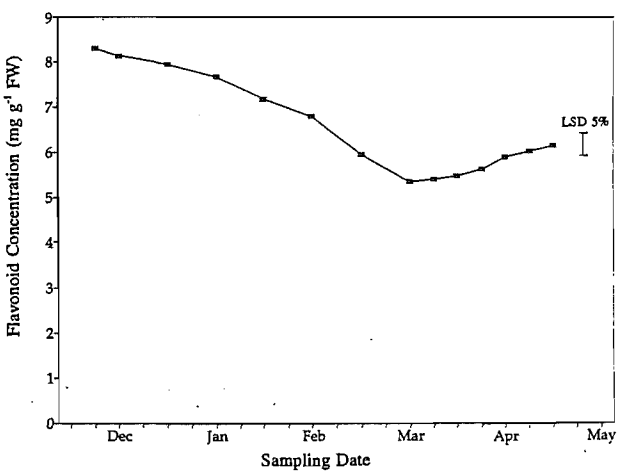


Figure 5.10: Changes in enzyme activities and flavonoid concentration in 'Granny Smith' apples during development

This drop was followed by a steady rise in activity returning to about 50% of the original level in 'Splendour' (410 pKat g^{-1} FW) and just over 25% in 'Granny Smith' (95 pKat g^{-1} FW).

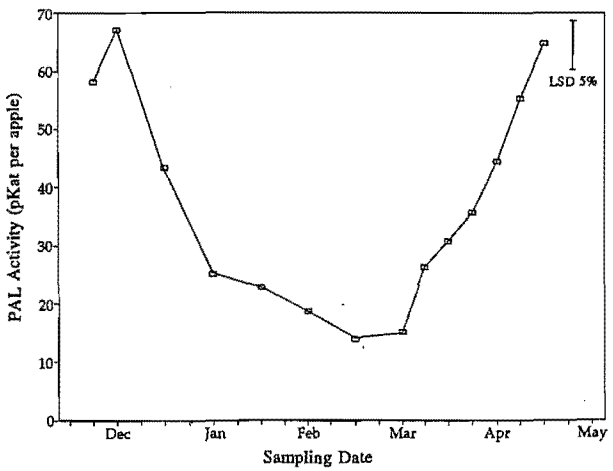
The total amount of CHI activity per apple showed a similar pattern of change to PAL although the drop in the first half of the season was not as marked, particularly for 'Splendour'. For 'Splendour' the average initial level of activity was 1185 pKat per apple and for 'Granny Smith' it was much lower at 500 pKat per apple. For 'Granny Smith' the major decrease in activity occurred in the first five weeks (Figure 5.12 b), and 'Splendour' showed a steadier, but slower drop right through to early March (Figure 5.11 b). In early March for 'Splendour' about 50% of the initial peak CHI activity remained and for 'Granny Smith' this figure was 25%, compared to the PAL activity values of 25% and 0% respectively. The level of CHI activity at the final harvest was also much higher in 'Splendour', at about three times the initial level (3628 pKat per apple). However, for 'Granny Smith' the final level was only slightly higher than the initial, at 614 pKat per apple.

(c) GT

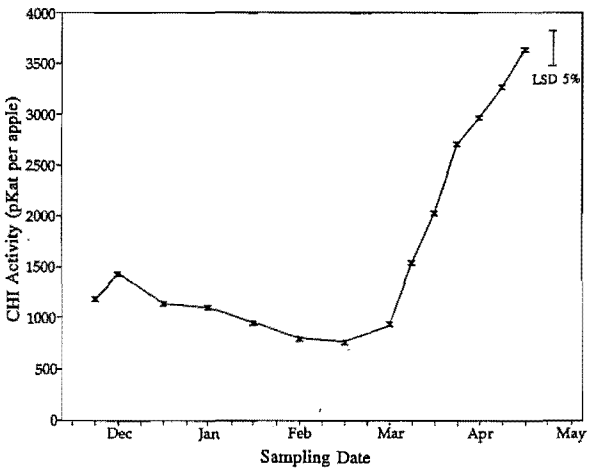
The patterns of change in GT activity, when expressed per gram FW, were similar to the other two enzymes with a few slight differences. As with the other enzymes the GT activity in 'Splendour' (Figure 5.9 c) was much higher than 'Granny Smith' (Figure 5.10 c) at all stages of development. However, the difference was greater than the other two enzymes at approximately six times higher initially and eight times higher by the final sampling. Initial levels of GT activity were 137 pKat g^{-1} FW in 'Splendour' and 23 pKat g^{-1} FW In 'Granny Smith'. 'Splendour' showed a slight increase (4%) in GT activity between the first and second samplings, although this was not significant, however no increase was observed for 'Granny Smith'. As with the other two enzymes there was a drop in activity through to early March, although this was not as great with 30% of the peak GT activity still present in 'Splendour' and 40% in 'Granny Smith'. In both cultivars the levels of GT activity rose from early March through to the final harvest. In 'Splendour' GT activity returned to close to original levels (141 pKat g^{-1} FW) and in 'Granny Smith' about 75% of the original activity was reached (17 pKat g^{-1} FW).

The total amount of GT activity per fruit showed a slightly different pattern of change. In 'Splendour' the initial level of CHI activity per apple was 206 pKat and there were no significant differences in activity up to early March. From mid-March there was a rise in activity which continued through to the final harvest (Figure 5.11 c). The final level of GT activity was approximately six times the initial level (1252 pKat per apple). A similar pattern of change was observed for 'Granny Smith' although the rise was not as dramatic (Figure 5.12 c). Initial activity per apple was 33 pKat and the final level of activity was only about 3.5 times this at 112 pKat. The difference between the level of CHI activity in 'Splendour' and 'Granny Smith' became more pronounced as the season progressed. Initially activity per apple in 'Splendour' was approximately six times higher but by late April it was 11 times higher.

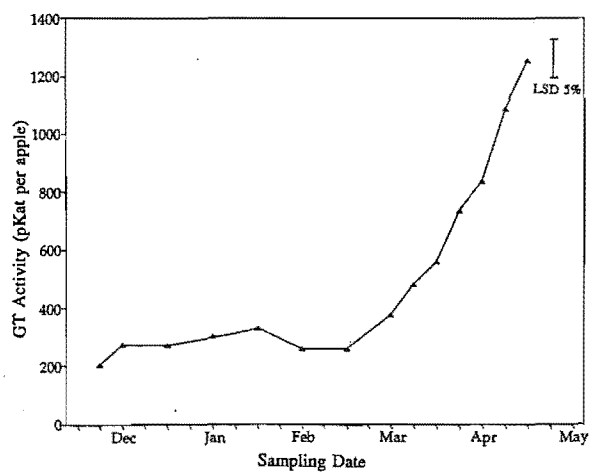
(a) PAL



(b) CHI



(c) GT



(d) Flavonoid Concentrations

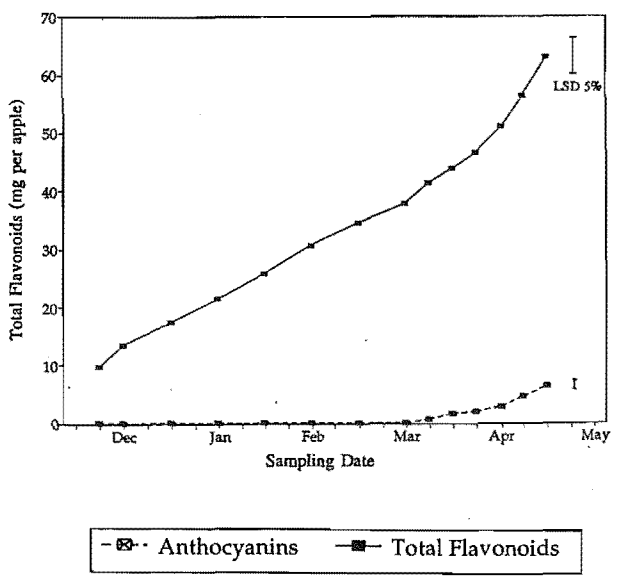
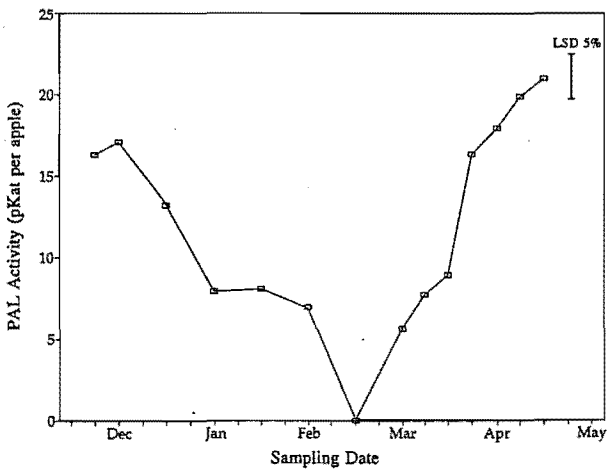
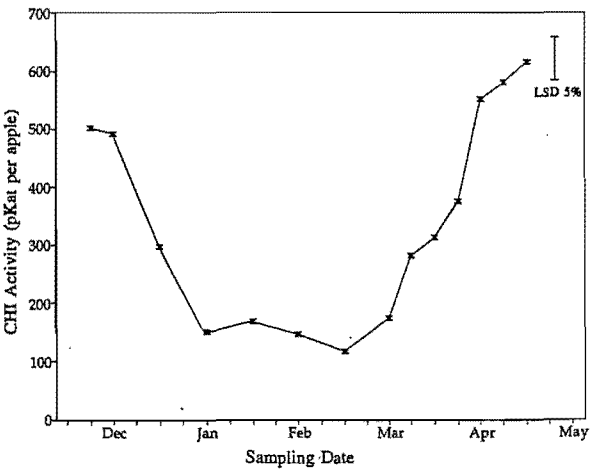


Figure 5.11: Changes in enzyme activity and flavonoid amount per apple in 'Splendour' apples during development

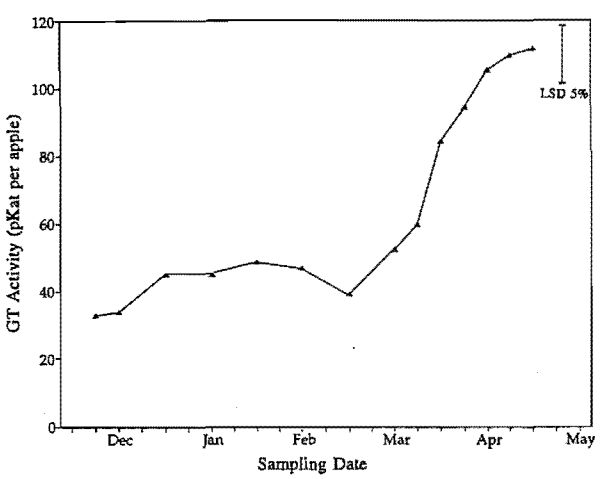
(a) PAL



(b) CHI



(c) GT



(d) Flavonoid Concentrations

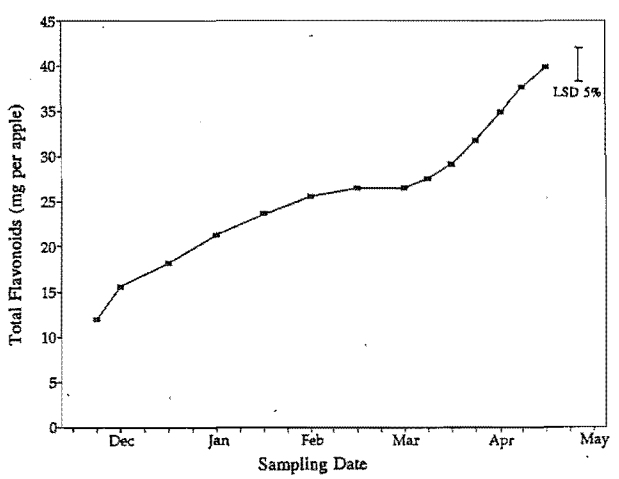


Figure 5.12: Changes in enzyme activity and flavonoid amount per apple in 'Granny Smith' apples during development

(d) Reductases

Attempts made to quantify the reaction products by HPLC were unsuccessful due to low concentrations of product and interference from other compounds. Thus, reaction products could only be estimated by the vanillin and butanol tests which did not yield information on specific products or accurate quantification. Although this assay was not precise enough to quantify enzyme activity it was performed to show general trends and the patterns of change for both reductases (dihydroflavonol- and diol-reductase) were similar to that of the other enzymes, in both cultivars. Immature fruit contained the highest reductase activity, which dropped to undetectable levels until early March when there was a rise in activity through to the final harvest. As with PAL and CHI, when activity was examined on the basis of amount per gram fresh weight the final enzyme activity was not as high as the original; in this case being approximately 75% for both cultivars. When activity was expressed in amount per apple, the activity was relatively steady until March and then rose steadily through to the end of the season. Reductase activity in 'Splendour' was significantly higher that of 'Granny Smith', at about four times higher initially and nearly six times higher at the final sampling.

(e) Flavonoid 3′-hydroxylase

Attempts to measure the activity of this enzyme were unsuccessful. This may have been due to unsuccessful extraction of the microsomal enzyme, or the activity of the enzyme was below the limits of detection of this the assay system. Limits of detection were not ascertained for the enzyme system so product may have been formed but at a concentration below detection levels. Using purchased standards the detection system provided clear separation of kaempferol and quercetin but not quercetin and myricetin (Figure 5.13) but the latter would not expected in this system.

Quantification of flavonoids

The patterns of change in the flavonoids were similar to those reported in Section Four with some slight differences in levels (Figure 5.9 d to 5.10 d). For 'Splendour' anthocyanin levels were close to zero before early March and then they rose steadily through to the final harvest in late April, reaching 1.10 mg g^{-1} FW. When total flavonoids (including anthocyanin) were examined, on a concentration basis, initial concentrations in the two cultivars were similar at 8.1 mg g^{-1} FW for 'Splendour' and 8.28 mg g^{-1} FW for 'Granny Smith'. These levels then dropped through to mid-March, to a low of 6.9 mg g^{-1} FW for 'Splendour' and 5.4 mg g^{-1} FW for 'Granny Smith'. In 'Splendour' there was a 7% rise in concentration between the first and second samplings, but this was not observed in 'Granny Smith'. In both cultivars the drop in levels were followed by a rise through to the final sampling which was more pronounced in 'Splendour'. Apart from the initial levels 'Granny Smith' fruit had lower pigment concentrations than 'Splendour' and this became particularly marked as the season progressed.

When expressed in terms of total flavonoids per apple both cultivars increased over the whole

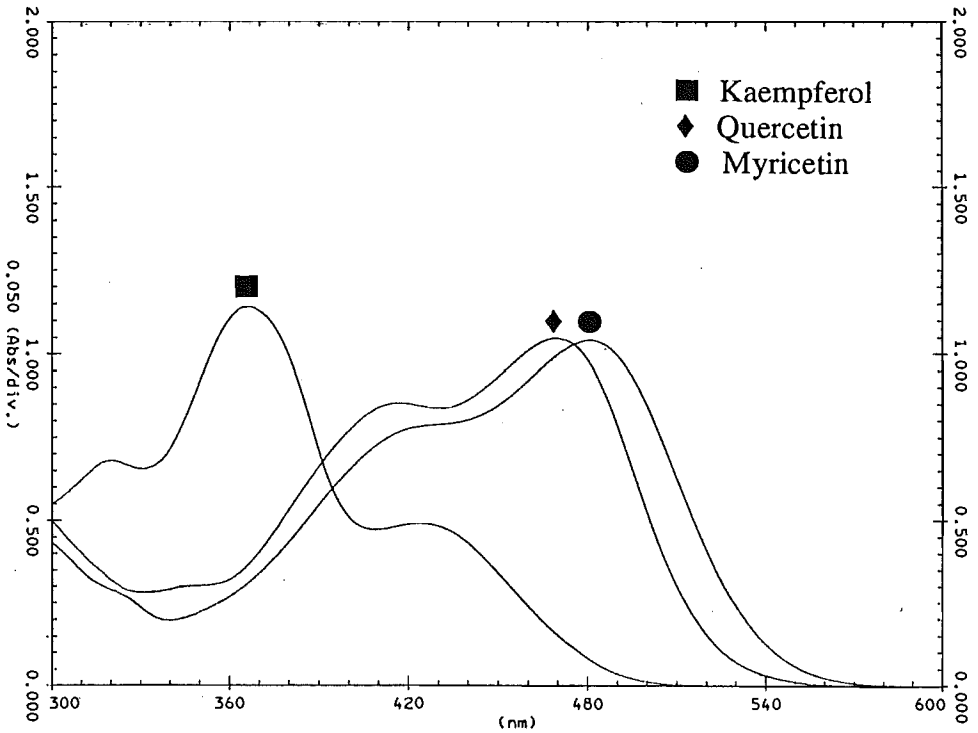


Figure 5.13: Absorption spectra for flavonol complexes with Naturstoffreagenz A

season, with a higher rate of increase over the last month (Figure 5.11 d & 5.12 d). In 'Granny Smith' there was a period of a month, from mid-February to mid-March, where there was little significant change in the flavonoid amount per apple. The final amount of flavonoids per apple in 'Splendour' (78.8 mg) was approximately double that of 'Granny Smith' (39.9 mg), although the initial levels were similar. Anthocyanins in 'Splendour' showed the same rapid increase though over a period of about six weeks (from mid-March to late April). In 'Splendour' the final amount of anthocyanin per apple was 9.7 mg.

Comparison of flavonoid levels with enzyme activities

The changes in activity of all three enzymes during development correlated with changes in total flavonoids for both cultivars, and anthocyanins for 'Splendour' (Table 5.7). Most showed significant correlation of enzyme activity with flavonoid accumulation, particularly during ripening. For 'Splendour' correlation between activity of all three enzymes and anthocyanin concentration was only significant when the data from last eight weeks were analysed; prior to this anthocyanin concentrations were close to zero.

PAL had the lowest activity of the three enzymes measured so it was used to estimate the possible amount of flavonoid able to be synthesised but these calculations did not take into account degradation of flavonoids, since this was an unknown factor. Based on the level of PAL activity large amounts of flavonoids could have been synthesised in the period from December through to March when flavonoid levels dropped, on a concentration basis. Theoretically, in 'Splendour' over the last eight weeks before

Table 5.7: Comparison of enzyme activities and flavonoid accumulation

Variables correlated	Correlation coefficient (r)	
	concentration	amount per apple
Splendour		
PAL vs Anthocyanin (all)	0.20	0.53*
PAL vs Anthocyanin[a]	0.98*	0.98*
PAL vs Total Flavonoid (all)	0.57*	0.04
PAL vs Total Flavonoid[a]	0.93*	0.99*
CHI vs Anthocyanin (all)	0.07	0.94*
CHI vs Anthocyanin[a]	0.91*	0.92*
CHI vs Total Flavonoid (all)	0.69*	0.79*
CHI vs Total Flavonoid[a]	0.81*	0.95*
GT vs Anthocyanin (all)	0.50	0.98*
GT vs Anthocyanin[a]	0.99*	0.99*
GT vs Total Flavonoid (all)	0.84*	0.89*
GT vs Total Flavonoid[a]	0.95*	0.99*
Granny Smith		
PAL vs Total Flavonoid (all)	0.81*	0.26
PAL vs Total Flavonoid[a]	0.94*	0.96*
CHI vs Total Flavonoid (all)	0.72*	0.33
CHI vs Total Flavonoid[a]	0.97*	0.95*
GT vs Total Flavonoid (all)	0.77*	0.90*
GT vs Total Flavonoid[a]	0.89*	0.93*

[a] During ripening only - from mid-March to late April
* Denotes correlation coefficient significant at p=0.05, n=14

harvest, 8.22 mg of flavonoid could be synthesised per gram FW yet only 1.97 mg g^{-1} actually accumulated. In 'Granny Smith', for the same period, 2.06 mg could theoretically be synthesised but only 0.49 mg g^{-1} accumulated.

When calculated on the basis of amounts per apple the theoretical accumulation of flavonoids in 'Splendour' was usually two to three times higher than the actual accumulation rate and sometimes up to eight times higher. At no stage was the theoretical rate lower than the actual rate. For 'Granny Smith' the theoretical accumulation rate was between 1.5 and 4 times the actual rate, although at the point where no PAL activity was detected there was a very small accumulation of flavonoids. However, as mentioned earlier, there may have been a small amount of activity at this point.

5.2.4 Discussion

Extraction of enzyme activity

The method used for extraction of the flavonoid enzymes from apple skin gave the highest levels of activity for all three major enzymes (PAL, CHI and GT). Many other methods were tested including preparation of acetone powders, vacuum infiltration of tissue, freeze-drying the skin, use of insoluble PVPP and other protective agents. However these methods gave lower or, in some cases, no enzyme activity and this was often indicated by brown, rather than green, extracts.

The levels of enzyme activity for the three flavonoid enzymes were lower than those reported for many other tissues such as flowers, cell cultures, seedlings and some fruit (Dixon *et al.*, 1988; Cheng & Breen, 1991; Kakegawa *et al.*, 1991; Zielslin & Ben-Zaken, 1991). Similar levels of activity to apples were found in some other fruits such as grapes and sweet cherry (Melin *et al.*, 1977; Kataoka *et al.*, 1983). These differences in enzyme activities probably relate to the time-frame in which the flavonoids are produced and the final concentrations of the flavonoids in the fruit. In apple skin the anthocyanins are synthesized over a period of six weeks, and the flavonoids over a longer period, whereas in flowers and seedlings pigment production often occurs in a matter of days or even hours. In addition the final anthocyanin levels in the highly coloured flowers usually studied is much higher. Thus, it is not unexpected that the apple skin had a lower level of enzyme activity.

Attempts were made to measure enzyme activity of the flavonoid 3′-hydroxylase since this is an important enzyme in apples due to the predominance of 3′,4′-flavonoids. Methods used previously were based on radioactively labelled compounds (Hagmann *et al.*, 1983; Stotz *et al.*, 1985) but these were not used due to expense and the time needed to synthesize the compounds. Measurements of activity were attempted using an adaptation of the Naturstoffreagenz A reagent used as a TLC spray. The increase in hydroxylation of the B-ring was matched by a shift in the absorption spectra maximum from 365 nm to 470 nm, which was easily measurable. Problems in detecting enzyme activity may have been due to problems with enzyme extraction since this enzyme is microsomal and only crude preparations were tested. Other methods needed to be trialled to determine if there was a better preparation method to obtain enzyme activity. The other problem may be in the levels of enzyme activity as these are fairly low in apples compared to flowers as discussed earlier. This assay system may be useful for detection of enzyme activity in flowers and other tissues where higher levels of activity are observed.

Determination of K_m values

No previous reports of the K_m for PAL from apple skin could be found but most reported values for other plants lie in the range 0.3×10^{-4} M to 1.5×10^{-2} M (Camm & Towers, 1973) and the values obtained for 'Splendour' and 'Granny Smith' apple skin fell well within this range. The values for the two cultivars were similar but not identical. It has been noted though that with a number of preparations there is no single value for K_m (Neish, 1961; Havir & Hanson, 1968; Marsh *et al.*, 1968).

Dixon *et al.* (1988) studied the chalcone isomerases from a number of different plant sources and

reported K_m values of between 1 μM, for *Petunia hybrida*, and 100 μM, for *Medicago sativa*. The values obtained for both 'Splendour' and 'Granny Smith' apple skin are within, but at the high end, of the range.

Analysis of PAL activity in different cultivars

The levels of PAL activity measured in New Zealand-grown apples were similar to many reported previously (Faragher & Brohier, 1984; Arakawa *et al.*, 1986; Kubo *et al.*, 1988). However the activities were lower than the levels reported for skin disks or light induced tissues (Faragher & Chalmers, 1977; Tan, 1979; Arakawa *et al.*, 1986). This is not surprising as PAL is induced by wounding (as in skin disks) and also by light, which leads to more rapid accumulation of flavonoids than in normal tissue. Blankenship and Unrath (1988) reported much lower levels of activity, indicating they may have lost some activity as the levels would not be sufficient to allow for normal pigment production.

There was no correlation between PAL activity and anthocyanin which is not unexpected as these are only a small group of flavonoids and no correlation was observed between anthocyanin and total flavonoids (Section Three) however there was a correlation between average PAL activity and total flavonoid concentrations. It is not really surprising that a better correlation was not obtained since PAL is not specific to the flavonoid biosynthetic pathway being involved also in the synthesis of other compounds such as chlorogenic acid. A better correlation may have been observed between PAL activity and total phenolics. Other enzymes specific to the flavonoid pathway, such as CHI and GT, may have given better indications of the rate of synthesis of the flavonoids and correlated with anthocyanins and flavonoids. Another explanation for the lack of correlation is possibly the pattern of anthocyanin accumulation which appears to vary between some cultivars. In a cultivar such as 'Red Delicious', which had high anthocyanin levels, a higher level of PAL activity might have been expected. However, the pattern of accumulation of anthocyanin, based on visual observation, was also different from many other varieties in that it was not restricted to the last month of development, during ripening. PAL activity was more constant throughout development of 'Red Delicious', matching the gradual accumulation of the anthocyanins rather than a rapid increase during ripening observed in varieties such as 'Splendour'.

PAL-IS

The fact that the levels of PAL-IS were similar in the two cultivars means that it could not cause the differences in the daily synthesis rate of the flavonoids ('Granny Smith' 0.28 mg day^{-1} and 'Splendour' 0.72 mg day^{-1}). Thus, these differences are probably directly related to the levels of enzyme activity. It would appear that PAL-IS was not involved in the regulation of anthocyanin biosynthesis by controlling PAL activity since there was no drop in level on ripening when the anthocyanins increased. Nor did it appear to be a controlling factor in the biosynthesis of other flavonoids since the only drop in PAL-IS occurred early in development when flavonoids were also dropping and there was still appreciable PAL

activity.

The role of regulation of PAL-IS on *in vivo* PAL activity is questionable (Blankenship & Unrath, 1988) and it is possible that PAL-IS may have no effect being located separately in the plant cell. Studies by Blankenship & Unrath (1988) also suggested that PAL-IS was not closely involved with the regulation of anthocyanin synthesis in apple skin. Similar PAL-IS levels were observed in 'Red Delicious' and 'Golden Delicious' apples. Tan (1979) has shown that PAL-IS may regulate the activity of PAL in the skin of whole apples although these studies involved wounding and light treatments and no reference was made to the effects during development. Thus, PAL-IS may have an involvement in controlling PAL levels in stress responses but does not appear to be the ultimate controlling factor in influencing the pattern of change in flavonoids, or anthocyanins, during development.

Substrate and sugar specificity of glycosyltransferase

The lack of glycosylation of the flavonoid intermediates supports the idea that glycosylation is a terminal step in flavonoid biosynthesis, which has been shown in many enzyme studies (Jourdan & Mansell, 1982). It also adds to the evidence for the existence of linear multienzyme complexes (Stafford, 1990). Stafford (1990) reported that some of the intermediates could be glycosylated for storage, but if they are ever to re-enter the main pathway hydrolysis must occur first since the enzymes only function with the aglycones. However, glycosides of dihydroquercetin are common in some gymnosperm tissues. Some types of UDP:flavonoid-3-O-glycosyltransferases are undoubtedly involved in these cases but so far none of the enzymes isolated from any plants accept dihydroflavonols as substrates (Stafford, 1990). As has been observed here apples are one of these and this is also backed up by the fact that small traces of dihydroquercetin were sometimes observed in apples but these were not glycosylated.

The hydroxylation pattern of the flavonol B-ring did not have a significant effect on activity with galactose as the sugar donor. However, quercetin showed much higher activity with glucose and xylose than did any of the other compounds and the levels of activity of quercetin with the different sugars matched the ratios of these compounds seen in apple skin at this stage of development. Thus, the pattern of glycosylation is dictated by the substrate specificity or activity of the glycosyltransferases and not the levels of the sugars present in the skin.

The anthocyanin cyanidin (3′,4′-OH), which is the only one occurring in apples, showed the highest level of activity. There was still high activity with pelargonidin (4′-OH) and delphinidin (3′,4′,5′-OH) indicating that the degree of B-ring hydroxylation did not significantly affect glycosylation as was observed with the flavonols. Therefore the fact that these anthocyanins are not present in apples is not due to an inability to glycosylate them, and this must be controlled at an earlier point in the pathway. Again, the levels of activity of cyanidin with the different sugars matched the pattern of anthocyanins seen in the skin, with cyanidin-3-galactoside 95% and cyanidin-3-glucoside approximately 5% of the total anthocyanin. Thus, the pattern of glycosylation appears to be a function of the substrate specificity or

levels of the enzyme(s) and not the levels of the various sugars in the fruit.

The ability to glycosylate methylated flavonoids appears to vary and may depend on the position of methylation. Isorhamnetin, which is quercetin methylated at the 3′ position, was glycosylated but rhamnetin (7 position) and malvidin (3′, 5′ methylation) had only very low glycosylation rates. Thus, even if methyltransferases were present in apples, many of the compounds might not be glycosylated and therefore not accumulated.

Further study is required to determine the actual number of glycosyltransferase enzymes present in apples. Genetical and biochemical investigations in *Silene* have shown the existence of allozymes each differing in substrate specificity not only for the sugar to be transferred, but also for the flavonoid acceptor (Steyns & van Brederode, 1986a & b; van Brederode *et al.*, 1987). However, in apples this would seem wasteful since many more enzymes appear to be present than are required for the compounds present. There may however be different allozymes for each of the sugars which will have activity with a range of different substrates to varying degrees.

Changes in enzyme activity during fruit development

The three enzymes studied in detail were selected for several reasons, the major one being that they represented an early (PAL), a middle (CHI) and a late (GT) stage in flavonoid biosynthesis. If these three enzymes change in a similar manner then it could be expected that the enzymes in between would also show a similar pattern of change during development.

Both 'Splendour' and 'Granny Smith' showed a similar pattern of change during development although both enzyme activities and flavonoid levels were higher in 'Splendour'. The rise in enzyme activity was much lower in 'Granny Smith' probably due to no anthocyanin biosynthesis and lower flavonoid levels. As expected there were high levels of enzyme activity present in immature fruit which would be necessary to synthesise the high levels of flavonoids also detected, but there was still an excess of activity. Although the levels of flavonoids then dropped through to March there was still significant enzyme activity but it did decrease. Thus, although the enzyme activities and flavonoid levels were correlated, they were not the only controlling factors and substrate may have been limiting or the activity of an enzyme not measured. Since little is known about the turnover of these enzymes it is possible that during this time there was little *de novo* synthesis and the activity measured was mainly residual. To determine more precisely what is happening the levels of *de novo* synthesis need to be established. This could be done by determining the relative level of PAL gene activity in the skin by estimating the amount of PAL mRNA. In some plants, including the apple (Tan, 1979), inactivators are present (Zucker, 1968; Creasy, 1976; Tanaka *et al.*, 1977) and in *Xanthium* the 'induction' of PAL was found to be due to a decreased rate of inactivation of the PAL molecule (Zucker, 1971). In this system there was continuous production of inactivator and changes in PAL levels were due to a change in rate of synthesis of the inactivator and not of an increased rate of synthesis of PAL. The drop in enzyme activity in apples did not appear to be the result of the action of inhibitors (PAL-IS or otherwise) since enzyme activities were

always additive.

Activity of all three enzymes was shown to correlate with flavonoid accumulation in both cultivars when examined in terms of concentration, although PAL correlated only during ripening. There was a correlation between enzyme activities and anthocyanin during ripening, but not for the whole season as anthocyanins were close to zero up to mid-March. Thus, activity of the enzymes is required for anthocyanin biosynthesis but activity does not guarantee it. This demonstrates further that the key step for anthocyanin biosynthesis is at the final stage of the pathway at "anthocyanin synthase". Poor correlations of PAL with total flavonoids over the early stages of development indicate its involvement in other biosynthetic pathways but correlation over ripening suggests that the increase in PAL was intimately involved in the increase in flavonoid biosynthesis and, in particular, the anthocyanins. One reason for the discrepancy between PAL and flavonoids in early development is that the very high PAL activity in immature fruit may have also been due to the biosynthesis of other compounds such as chlorogenic acid. This was noted to be high in immature fruit and dropped steadily over the season, similar changes were also noted by Walker (1962). The closer relationship between PAL and flavonoids at the end of the season might indicate that there were fewer changes in these other compounds.

Only PAL has been studied previously in intact apple fruit and the results obtained in this study are similar to those obtained by Kubo *et al.* (1988) for the development of four different cultivars. These workers also noted that in a non-red apple ('Golden Delicious') PAL activity increased on ripening as was observed with 'Granny Smith' in this study. Blankenship & Unrath (1988) also studied changes in PAL during maturation and showed an increase in PAL, followed by a drop in both 'Red Delicious' and 'Golden Delicious'. The drop was not observed here but this may be that the last sample was taken before this stage was reached. Anthocyanin levels had reached a plateau in the 'Red Delicious' fruit used by Blankenship & Unrath (1988) but were still increasing in 'Splendour' measured in this study. The increase in anthocyanin on ripening may be linked to the development of the climacteric. A number of workers have studied the effect of ethylene on PAL and the accumulation of anthocyanins in the skin of red apples (Chalmers & Faragher, 1977b; Faragher & Brohier, 1984; Murphey & Dilley, 1988). Ethylene increased PAL activity and anthocyanin accumulation in unripe apples but not ripe fruit indicating a possible role in initiation of anthocyanin biosynthesis by increasing the level of PAL in the skin. However, Knee (1972) noted that there were marked differences between the pigment changes during the ripening of attached and detached fruits. The development of fruit attached to the tree was slower but the pigment changes were more extensive. Anthocyanin also increased during the pre-climacteric phase under some conditions and in some cultivars, thus its metabolism may not be affected by the climacteric.

There have been some studies of changes in activities of the flavonoid enzymes during the development of various fruits, although these have mainly concentrated on PAL. A wider range of enzyme activities have been studied in flowers but these are difficult to compare with changes in fruit since the developmental patterns are totally different. Similar patterns of change in enzyme activity have been observed in developing strawberry (*Fragaria* ×*ananassa* Duch.) fruit where there were two peaks

in PAL activity (Cheng & Breen, 1991). There was one peak of activity in immature green fruit, coinciding with an increase in flavonoids other than anthocyanins, and the second peak of PAL activity was concomitant with the rise in anthocyanin. The rise in activity during ripening was due to *de novo* enzyme synthesis (Given *et al.*, 1988b). The change in activity of UDP-glucose:flavonoid-3-*O*-glucosyltransferase has been shown to parallel that of PAL (Given *et al.*, 1988a). Although the levels of PAL activity in strawberry fruit were 10 to 20 times higher than in apple fruit the levels are almost directly comparable when the time frame of pigment synthesis is taken into account. Anthocyanin accumulation in strawberry takes place over a period of two to three days compared to about six weeks in apples. In sweet cherry (*Prunus avium* L. var Bigarreau Napoléon) fruit PAL levels were very high in immature fruit dropping to very low levels then rising slightly on ripening (Melin *et al.*, 1977). Concomitant variations in PAL activity and in flavonoid concentration were found during growth but the increase in PAL activity on ripening did not appear to be directly related to anthocyanin synthesis. A similar pattern of change was observed during the development of grapes with high PAL levels in immature fruit dropping, and then rising during anthocyanin accumulation (Kataoka *et al.*, 1983). However, the second rise in PAL activity occurred only in black varieties not in white varieties. A good correlation was observed between PAL and anthocyanin concentration in black grapes. PAL has also been shown to be correlated with flavonoid synthesis in strawberry (*Fragaria vesca* var. 'Alpine') leaf disks (Creasy, 1968a). A direct relationship was observed between PAL activity and the rate of naringenin glycoside accumulation in developing grapefruit (*Citrus paradisi* Macf.) (Maier & Hasegawa, 1970). The pattern of PAL activity during the development of pear fruit was slightly different, although at a maximum in immature fruit it then dropped during the growth of the fruit and did not rise again (Melin-Moulet & Hartmann, 1978). Thus in general, PAL activity is at a maximum in very young fruits and falls rapidly during growth and only in those fruit that accumulate anthocyanins, or other flavonoids, during maturation is there a second rise in activity (Macheix *et al.*, 1990). Enzyme changes in apple fruit skins were in agreement with this observation.

Tissues of fleshy fruits commonly have high levels of flavonoids during development but accumulate anthocyanins only near maturity and these changes are matched by changes in activity of at least some of the enzymes of flavonoid biosynthesis. In most fruits, including apples, the pattern of flavonoid accumulation appears to be developmentally regulated by the control of PAL activity and the enzymes specific to the flavonoid biosynthetic pathway. Other factors, such as inhibitors like PAL-IS or compartmentation, do not appear to be responsible for the changes occurring in flavonoid levels during development. These factors may still have an influence on the concentration of the flavonoids but not the pattern of change. PAL activity is a prerequisite for anthocyanin accumulation but it does not guarantee that synthesis will occur, since in green fruit 'Granny Smith' also have appreciable PAL levels. However, PAL is also necessary for accumulation of the other flavonoids which do increase on ripening in both cultivars.

Control of flavonoid biosynthesis

So, where is the control point(s) in the flavonoid biosynthetic pathway in apples? Is enzyme activity limited by the concentration of the substrate or by the level of enzyme? PAL is frequently considered the rate-limiting step in some systems (Zucker, 1972; Camm & Towers, 1973; Hahlbrock *et al.*, 1976). Chalcone synthase (CHS) has also been reported as rate-limiting in the biosynthesis of flavonoids (Knogge *et al.*, 1986; Schmelzer *et al.*, 1988). On the other hand, Margna (1977) has suggested that the level of phenylalanine may be the rate-limiting factor. Feedback inhibition and enzyme repression may also be more efficient ways to 'turn off' or reduce an enzyme activity. The sequestration of important substrates, such as phenylalanine, from active metabolic sites of a sequence could be another mechanism. Conversion of phenylalanine to expensive secondary pathways without regulation does not seem likely (Stafford, 1990) as it would be inefficient for the plant.

PAL had the lowest activity of the three enzymes measured but did not correlate directly with flavonoid accumulation at all stages of development. The changes in the levels of the flavonoids may not coincide with the changes taking place in the level of all polyphenols. Thus, it is still possible that the total production of phenylpropanoid compounds in apples is still positively correlated with changes in PAL activity throughout development since activity of PAL in many plants is correlated with simultaneous changes in the rate of accumulation of flavonoids and related phenolics (Margna, 1977). This suggests a possible causal relationship between the two and it has often been postulated that PAL is the main limiting factor in the biosynthesis of flavonoids, cinnamic acids and other phenylpropanoids (Zucker, 1972; Camm & Towers, 1973).

There are many cases where the potential capacity of available PAL to deaminate phenylalanine is much higher than would be expected from the amounts of phenolics synthesised (Ahmed & Swain, 1970; Maier & Hasegawa, 1970; Swain & Williams, 1970; Laanest & Margna, 1972). At all stages of apple development PAL activity far exceeded the amount of flavonoids produced both on the basis of concentration and amount per apple. The lowest ratio, from concentrations, of possible flavonoids accumulated (based on PAL activity) to actual flavonoids accumulated was 4:1, in ripening fruit. At most stages the ratio was higher than this, with significant PAL activity yet no flavonoid production. Even calculated on amount per apple the theoretical rate was always higher, usually about double actual accumulation. Thus, it seems that there are no pools of intermediates after PAL available for use in flavonoid synthesis under normal conditions and PAL activity is required. However, when making these calculations there are a number of other factors to take into consideration. The measured activity may not be the true rate *in vivo* since factors such as inhibitors and the pH may lower the actual activity. The calculations did not account for any degradation of the flavonoids, although it is highly unlikely that a high rate of degradation would occur. Many flavonoids have been shown to be relatively stable and in most cases degradation has been reported to be relatively low if occurring at all (Dangelmayr *et al.*, 1983; Zenner & Bopp, 1987) and thus degradation could only account for a small part of the difference. PAL is also involved in the biosynthesis of a range of other secondary products and only part of its activity

may be available for flavonoid biosynthesis. PAL is involved in the synthesis of chlorogenic acid which is present in high concentrations in apple skins (Walker, 1962; Coseteng & Lee, 1982; Burda *et al.*, 1990). Thus, part of PAL activity will be required for the synthesis of this and other compounds. Cheng & Breen (1991) reported discrepancy between phenolics and PAL activity in developing strawberry fruit and the difference between *in vivo* and *in vitro* PAL activity may have been responsible. Measurement of PAL activity has been reported to be extremely variable when activity is high, such as in young fruit of pear (Billot *et al.*, 1978), cherry (Melin *et al.*, 1977) and apple (Kubo *et al.*, 1988).

Isozymes are known for PAL and there may be a specific isozyme for the flavonoid pathway the activity of which may be limiting. Two isozymes of 4-coumarate:CoA ligase have been demonstrated in soybean cell cultures, one being concerned with the lignin pathway and the other flavonoids (Hahlbrock & Grisebach, 1979). In strawberry there was a parallel rise in PAL activity and anthocyanin level although the apparent excess of PAL indicated this may not have been the rate-limiting enzyme (Cheng & Breen, 1991). However, it was postulated that PAL may be a key factor controlling the channelling of phenylalanine into phenolic synthesis and hence flavonoid biosynthesis.

Despite these various considerations it is possible that the changes in PAL do not actually influence the rate of accumulation of flavonoids and other polyphenols. It is probable that the deaminating capacity of the plant is always sufficient to consume all the phenylalanine surplus to protein synthesis (Margna, 1977). Therefore if there is a shift in the accumulation of flavonoids it may not be the result of the controlling action of PAL but may be due to a change in the supply of the primary substrate, i.e. phenylalanine, to the enzyme. There are several pieces of data that help to support this hypothesis. Exogenous supply of phenylalanine has been shown to increase the accumulation of the flavonoids in a number of plants (Zucker, 1965; Grill, 1965 & 1967; Creasy, 1971). The levels of free phenylalanine are also low in many plant tissues (Pegg & Sequeira, 1968; Higuchi & Shimada, 1969) but there are no reports on the levels in apples. Inhibitors of protein synthesis have been found to stimulate anthocyanin formation (Faust, 1965; Wagner *et al.*, 1967) presumably by increasing the amount of phenylalanine available for flavonoid biosynthesis. However, these inhibitors have also been shown to inhibit anthocyanin formation in some plants (Lange & Mohr, 1965; Stafford, 1966; Scherf & Zenk, 1967), but this may have been the result of inhibition of *de novo* synthesis of critical enzymes in the flavonoid pathway (Chalmers & Faragher, 1977a). Without further investigation it is not possible to determine whether the level of phenylalanine is the rate-limiting step in the biosynthesis of flavonoids in apples.

Phenolics are generally viewed as being formed from phenylalanine arising directly from the shikimate pathway and unused in proteins, but it may also be of secondary origin, arising from the turnover and catabolic degradation of proteins (Margna *et al.*, 1989). In some plants there is a balanced relationship between protein metabolism and the formation of flavonoids and cinnamic acid derivatives (Margna, 1977). In buckwheat (*Fagopyrum esculentum* Monench) cotyledons more than half the total phenolics formed were from phenylalanine of secondary origin (Margna *et al.*, 1989). Thus the portion of phenylalanine becoming available for the formation of polyphenols is determined by the extent of its

utilization in the biosynthesis of new proteins and the level of release from protein turnover (Figure 5.14). Further experimentation is required to determine if this makes a significant contribution in apples. The possible increase in flavonoids during ripening could possibly be due to a drop in the rate of protein synthesis and/or an increased rate of degradation of the proteins, leading to an increase in available phenylalanine. However, the total protein content of apples is very low compared to many other plants and any contribution of secondary phenylalanine may not be significant.

Phenylalanine, leading to 4-coumaroyl-CoA, is only one of the precursors required for flavonoid synthesis with malonyl-CoA also required. The supply of this has been overlooked in the study of the regulation of flavonoid synthesis and its concentration at the site of synthesis is unknown (Stafford, 1990). Thus, it is possible that the level of malonyl-CoA could have an influence on flavonoid biosynthesis. Malonyl-CoA is the key intermediate in fatty acid biosynthesis which includes the biosynthesis of wax and cutin in apple skins and thus there may be some competition for the substrate between the two pathways. The formation of malonyl-CoA is by carboxylation of acetyl-CoA by acetyl-CoA carboxylase, a multienzyme complex (Goodwin & Mercer, 1983). This may be a possible control point in determining the availability of malonyl-CoA for flavonoid biosynthesis. Further investigation is required, supplying exogenous malonyl-CoA, to determine if this is limiting.

CHI activity was relatively high at all stages of apple development and thus was not a limiting factor. This level of activity would guarantee the 2*S* configuration of the flavanone which is important for biosynthesis (Beggs *et al.*, 1986). High CHI activity compared to the other flavonoid enzymes, such as PAL and CHS, has been reported for other plant tissues (van Weely *et al.*, 1983; Cosio & McClure, 1984; McCallum, 1989 & 1990b). Similarly GT activity was also reasonably high at all stages of development and even if the total theoretical amount of flavonoids, based on PAL activity, were

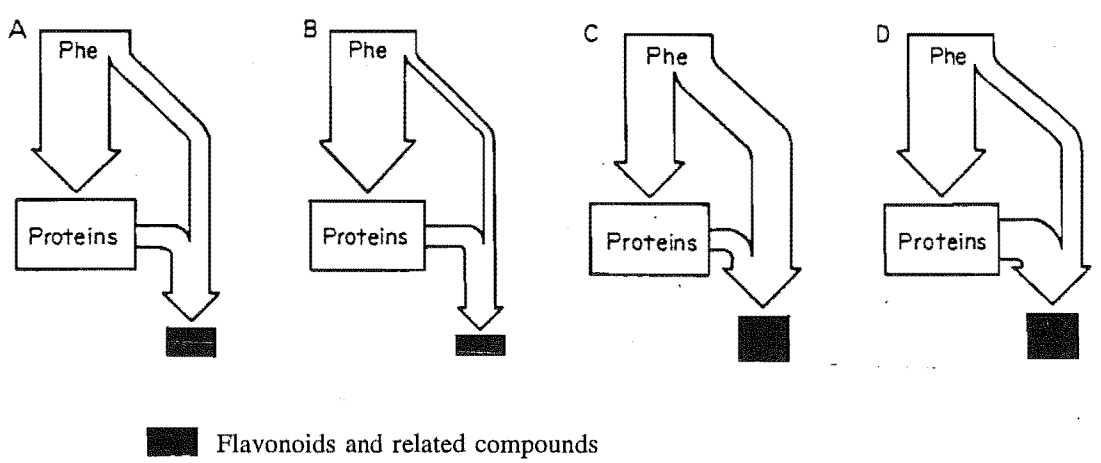


Figure 5.14: Balanced relationship between protein metabolism and phenylpropanoid accumulation as a reflection of changes in the distribution of phenylalanine between proteins and polyphenols. A-initial state; B-protein biosynthesis enhanced; C-protein biosynthesis repressed; D-protein turnover accelerated; Phe-phenylalanine (From Margna, 1977).

accumulated all would be glycosylated. Thus, based on enzyme activity, no aglycones would be expected to be present at any stage of apple development and this was the case. Thus, lack of anthocyanins in immature fruit is not the result of the degradation of the aglycone due to the inability to glycosylate it, as postulated by Chalmers *et al.* (1973).

Chalcone synthase may be an important regulatory step in the biosynthesis of the flavonoids in apples. It is likely that CHS activity would change during development in a similar manner to CHI, as has been reported in other tissues (Stafford, 1990). However, its level of activity may be the key factor in controlling the level of flavonoid production by being the rate-limiting step. A correlation was found between CHS activity and the rate of flavonoid accumulation in oat leaves and it was concluded that chalcone synthase was the rate-limiting enzyme of the pathway (Knogge *et al.*, 1986). However, Brödenfeldt & Mohr (1988) showed that, in the case of phytochrome-mediated flavonoid production in oat, CHS was not limiting and control was at some level beyond enzyme induction. Investigation of CHS activity is required in apples to determine if this is the rate-limiting step. There has been a recent report of an assay of CHS by HPLC (Zuurbier *et al.*, 1993) but based on the activities of the other flavonoid enzymes it is unlikely that this assay would be sensitive enough to detect activity in normal developing apple tissue. This assay was developed for *Petunia* which has much higher enzyme activities. Thus, to study developmental change in activity of this enzyme in apples a radioactive assay system would have to be used but due to its cost it was beyond the scope of this study.

The enzyme complex "anthocyanidin synthase" is important in the induction of anthocyanin biosynthesis but the other enzymes may also have to increase to allow sufficient flow of intermediates. Whether induction of all these enzymes is due to a common regulatory mechanism or whether one triggers the other is unknown. It is unlikely that the enzymes after CHS would be control points, since if they were limiting accumulation of intermediates would be observed. The interaction of inhibitors, such as PAL-IS, and feedback effects must not be excluded.

Another possible control mechanism is allosteric regulation of flavonoid enzymes via feedback inhibition, if the enzyme is in contact with the end products in the intact cell. Examples of end product, and intermediate, inhibition have been shown in a range of enzyme preparations from different sources (Stafford, 1990). PAL is well known to be inhibited by its product, cinnamate (Jones, 1984). Chalcone synthase from rye (*Secale cereale* L.) was inhibited by an isovitexin glycoside as well as apigenin, luteolin, naringenin and eriodictyol (Peters *et al.*, 1988). In *Daucus carota* CHS inhibition occurred only with naringenin (Hinderer & Seitz, 1985), and the end product kievitone inhibited CHS in *Phaseolus vulgaris* (Whitehead & Dixon, 1983). End product inhibition of chalcone isomerase has also been reported. Kievitone inhibited CHI in *Phaseolus vulgaris* (Hahlbrock *et al.*, 1970), and kaempferol and quercetin were shown to be inhibitors also in bean (Dixon *et al.*, 1982). Work with oat (*Avena sativa* L.) preparations showed no end product inhibition with the natural products so it may not be a general phenomenon (Stafford, 1990). Furthermore, if the flavonoid enzymes are organised as a multienzyme complex and products accumulate within vesicles or portions of the endoplasmic reticulum with the critical

enzymes on the cytoplasmic side such regulation would not be expected to occur *in vivo* (Stafford, 1990). Although PAL is not directly involved in the multienzyme complex, postulated by Stafford (1990), metabolic channelling within membranes might minimise the concentration of cinnamate and inhibition *in vivo* may be negligible (Noé *et al.*, 1980).

Differences in flavonoid accumulation in 'Splendour' and 'Granny Smith' are reflected by differences in enzymes activities in the two cultivars. Daily synthesis rates of flavonoids in 'Splendour' were two to four times than in 'Granny Smith' and likewise in 'Splendour' the activities of the three enzymes measured were between two and six times higher. Therefore the difference appears to be in a factor(s) controlling the whole pathway rather than a specific change in a single enzyme or for example reduced substrate availability due to lower PAL activity. Although the actual amount of flavonoid accumulated does not relate directly to enzyme activity the trends are the same in both cultivars, with approximately one quarter of the flavonoids synthesised (based on PAL activity). Thus, the level of flavonoids is likely to be a function of the enzyme activity rather than a difference in degradation rate or variation in the levels of inhibitors.

5.3 Further elucidation of the regulation of flavonoid biosynthesis

5.3.1 Introduction

5.3.1.1 Elucidation of flavonoid biosynthesis by the use of mutants

A large number of flavonoid pathway mutants can be found especially in *Zea mays* and *Petunia hybrida* (Stafford, 1990) and these mutants accumulate intermediates behind a mutant block in the biosynthetic pathway. These mutants have been useful in the elucidation of the steps in the flavonoid biosynthetic pathway and mutants have been found for each step in flavonoid biosynthesis from chalcone formation up to complex modifications of the anthocyanin (Heller & Forkmann, 1988). During this study no such mutants were observed in apples, with all cultivars accumulating all of the observed end products without major differences in hydroxylation or glycosylation patterns. Quercetin glycosides and proanthocyanidins were present in significant concentrations in all cultivars (Section Two and Three). There was no cultivar that showed accumulation of intermediates, such as dihydroquercetin or eriodictyol chalcone, and in no cultivar was there a lack of one of the normal flavonoid classes. The presence of such compounds could have been useful in determining the point at which 3′-hydroxylation occurs or given information on the control points in the pathway. Acyanic mutants are common in flowers and have been useful in elucidating anthocyanin biosynthesis but these have not been observed in apples. Cyanidin glycosides were present in all genotypes and species, although in some cases only in trace amounts in wounded fruit or those exposed to high light levels or other stresses. Anthocyanins were also often observed in immature fruit of normally non-red cultivars. Normally non-red cultivars such as 'Granny Smith' are not due to deletion of the anthocyanin biosynthesis gene(s), encoding for the enzymes converting leucocyanidin to cyanidin, since these fruit are capable of anthocyanin production under certain conditions. It is possible that from time to time mutations do occur in the flavonoid biosynthetic pathway in apples but these would not be seen in commercial cultivars since such mutations would ultimately affect anthocyanin formation and would not be selected due to poor colouration.

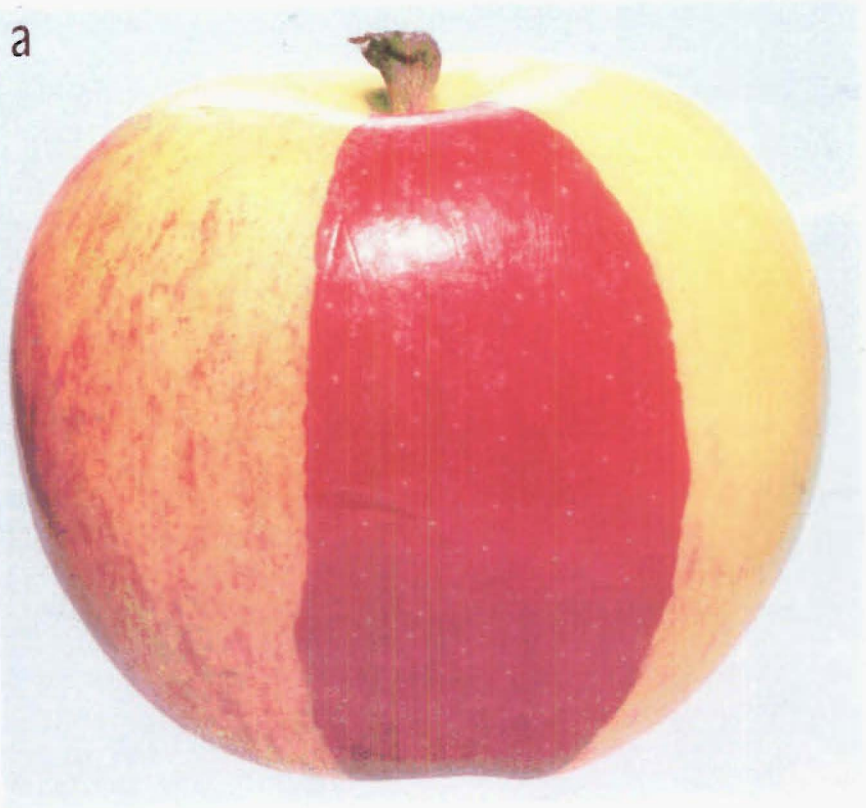
The only mutation frequently observed in apples is colour sports and most of these are chimeral in nature (Dermen, 1960); that is mutations or abnormal distribution of the chromosomes during development of the fruit which affect one cell and all its descendants (Dermen, 1948). One such chimera commonly observed in apple fruit is a darker red stripe on a green or lighter red background typical of the original fruit (Plate 5.1). In fruit with intense coloured fruits sharply delimited dull-colour sectors have been reported occasionally (Dermen, 1960) and the occurrence and nature of these chimeral sports has been discussed in detail by Dermen (1948 & 1960). Such mutations may yield useful information on the flavonoid biosynthetic pathway and give some clues as to the control of a specific compound, such as the anthocyanin in the case of red stripes. For example, is such the change in colour due solely to the induction of the "anthocyanin synthase" step(s) and hence anthocyanin production or is the whole pathway

Plate 5.1: Examples of chimeral stripes on apple skins

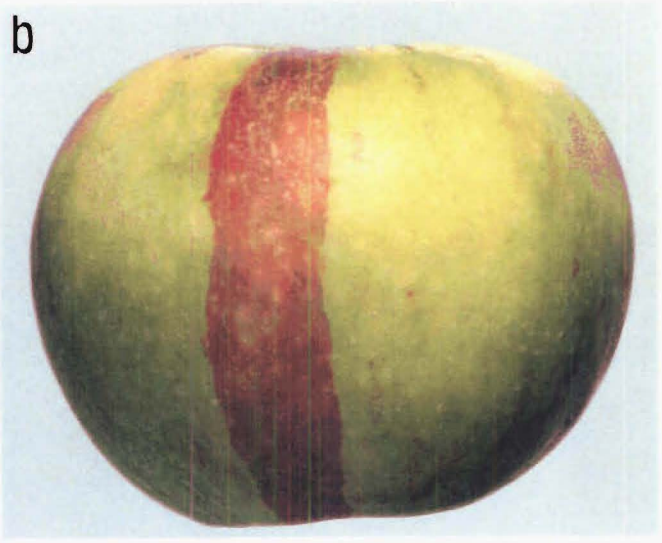
(a) 'Regal Gala'

(b) 'Cox's Orange'

a



b



affected altering the concentrations of flavonols and proanthocyanidins? There may also possible be a change in the pattern of glycosylation of the other flavonoids with the induction of cyanidin galactoside. These possibilities were examined by analysis of the flavonoid composition in the different coloured skin sectors.

5.3.1.2 UV induction of flavonoids

The development of post-harvest treatment regimes that will modulate and generate desirable apple skin colour without effecting the other characteristics of the fruit are important considerations in capturing and developing competitive markets (Dong *et al.*, 1994). Although genetic engineering provides an opportunity that may eventually influence fruit colour the time frame of 10-15 years to deliver transgenic fruit crops means there will be limited flexibility to accommodate short-term market trends. Thus, non-chemical and non-toxic manipulation of fruit colour at the post-harvest level is still a highly desirable goal. An understanding of the influence of external factors, such as light, on fruit colour may also provide information on the control of anthocyanin production. This could broaden our understanding of flavonoid biosynthesis and might be applicable to genetic manipulation.

Many flavonoids can be considered to be constitutive in plants, their appearance being controlled by endogenous factors during normal development (Stafford, 1990). However, in many cases flavonoid synthesis is induced by external factors and the best studied cases of induced flavonoid synthesis is photocontrol. Conversely, proanthocyanidin pathways appear to be entirely constitutive, although they can sometimes be increased by stress (Stafford, 1990). Photocontrol of flavonoid synthesis has been reviewed by Beggs *et al.* (1986) and in almost all cases the effect of light on total flavonoid or anthocyanin amount was studied.

There has been some study of the effect of light on flavonoid biosynthesis in apples. Anthocyanin biosynthesis is a light dependent process and hence apples kept in the dark or low light do not redden. Different apple cultivars redden to varying degrees depending on a minimum energy requirement (Proctor, 1974). It has been shown that white light plus UV at 312 nm produced four times the anthocyanin levels of white light alone (Arakawa *et al.*, 1986). It has also been shown that shading the fruit with UV cut-off film inhibited anthocyanin formation in red cultivars (Kubo *et al.*, 1988). However, all these studies have examined the effect of light on anthocyanins and not the other flavonoids. Quercetin glycosides were twice as high in the peel from the sun side of 'Golden Delicious' fruit compared to the shade side (Workman, 1963), however the increase in flavonols was not always accompanied by anthocyanin formation and reddening. Thus, increased UV light may also stimulate flavonol formation which is not surprising due to the close biosynthetic relationship between these compounds.

How does light stimulate anthocyanin synthesis? There is evidence that the enzymes, in particular PAL, in the biosynthetic pathway to anthocyanins are light-inducible which has received the most study. White light plus UV produced maximal PAL activity in 'Fuji' and 'Starking Delicious' (Arakawa *et al.*,

1986). The reddening of apple skin also involves the induction of additional enzyme(s) between leucocyanidin and cyanidin glycoside. However, since little is known about the enzyme(s) the details of how this is controlled has not been determined.

It is not known to what extent activation of the photoreceptors might influence the formation of different flavonoid patterns (Heller & Forkmann, 1988). Changes might be observed in the ratios of anthocyanin to flavonol and/or proanthocyanidin, or the range of glycosides observed. Thus, in addition to greater anthocyanin accumulation, and therefore reddening, these changes could have a significant affect on colour by changing copigmentation ratios or self-association factors. Changes in flavonoids were examined in apple skin stimulated with UV light and compared to green fruit from the centre of the tree not exposed to UV light. Naturally reddened fruit was compared to check that artificial stimulation of detached apples was not significantly different from the natural effects of UV light on fruit on the tree over a longer period of time.

5.3.2 Materials and methods

Materials

Apples of different cultivars with red stripes were obtained from the Lincoln University Orchard and HortResearch Havelock North Research Orchard (the fruit were not necessarily at maturity).

'Royal Gala' apples for the UV induction experiment were obtained from HortResearch Orchard, Auckland.

Methods

Analysis of flavonoids in chimeral stripes

The skin from the red strips were carefully cut out of the fruit taking care to scrape off all underlying flesh with a sharp scalpel. A sample of normal skin tissue adjacent to the stripe was also taken for analysis. Skin samples were frozen in liquid nitrogen and stored at -80°C prior to analysis. Flavonoids were extracted and analysed by HPLC (method using the variable wavelength detector) as in Section 2.2.

UV induction of flavonoid production

Three samples of 'Royal Gala' apples from each of the following groups were taken for analysis: (i) mature, non-red apples taken from centre of the tree for direct analysis of flavonoid content; (ii) mature, naturally reddened apples from the outside of the tree for direct analysis of flavonoid content; (iii) mature, non-red apples taken from the centre of the tree for UV induction and subsequent flavonoid

analysis.

UV irradiation of the apple sample (iii) was carried out by Dr. Arend Kootstra (HortResearch, Mt Albert Research Centre, Auckland, New Zealand). Green apples were irradiated with UV light, with a fluency of 150 $\mu W\ cm^{-2}$, for 48 hours at 17°C and humidity controlled at 50%. Samples were placed on a rotating disc to ensure even application of light. The skin, from two fruit for each sample, was removed immediately and frozen, as outlined above. The flavonoids were extracted by the methods outlined in Section 2.2. Identification and quantification of the individual compounds was also carried out by the improved HPLC method given in Section 5.2.1.

5.3.3 Results

Analysis of the flavonoids in chimeral stripes

There was an obvious visual increase in red colour, in the stripped skin compared to the normal tissue; this was the result of increased anthocyanin but in addition the other flavonoid groups (flavonols and proanthocyanidins) also increased in concentration (Figure 5.15). Increases in the flavonols and proanthocyanidins, although not as dramatic as the anthocyanins, were still significant. In two of the cultivars ('Cox's Orange' and 'Granny Smith') anthocyanins increased from nothing to over 0.1 mg g^{-1} and in 'Splendour' they increased 3.4 fold and 5.5 fold in 'Red Delicious'. The extent of the rise in flavonols and proanthocyanidins varied between the different cultivars from approximately one and a half to a three-fold increase but within a cultivar the increases in flavonols and proanthocyanidins were similar. There was only one exception, 'Red Delicious' where the proanthocyanidins only showed a marginal increase while the anthocyanins and flavonols increased threefold.

The composition of the flavonoids in the striped and normal skin was similar and the increase in concentrations of the flavonoid groups were not attributed to the appearance of new compounds. The relative percentages of the individual compounds were similar in the two tissues (Table 5.8).

UV induction of flavonoids

Appearance of the two groups of reddened fruit (UV-induced and naturally reddened on the tree) was the same being a similar bright red shade which was markedly different than the green control fruit. The concentrations of all three flavonoid groups were similar in the UV-induced and the naturally reddened 'Royal Gala' apple skin and these were both significantly higher than in the control fruit (Figure 5.16). When the green apple off the tree (control) was compared to the UV-treated sample it was noted that, as expected from visual appearance, there was a dramatic rise in the anthocyanin concentration from

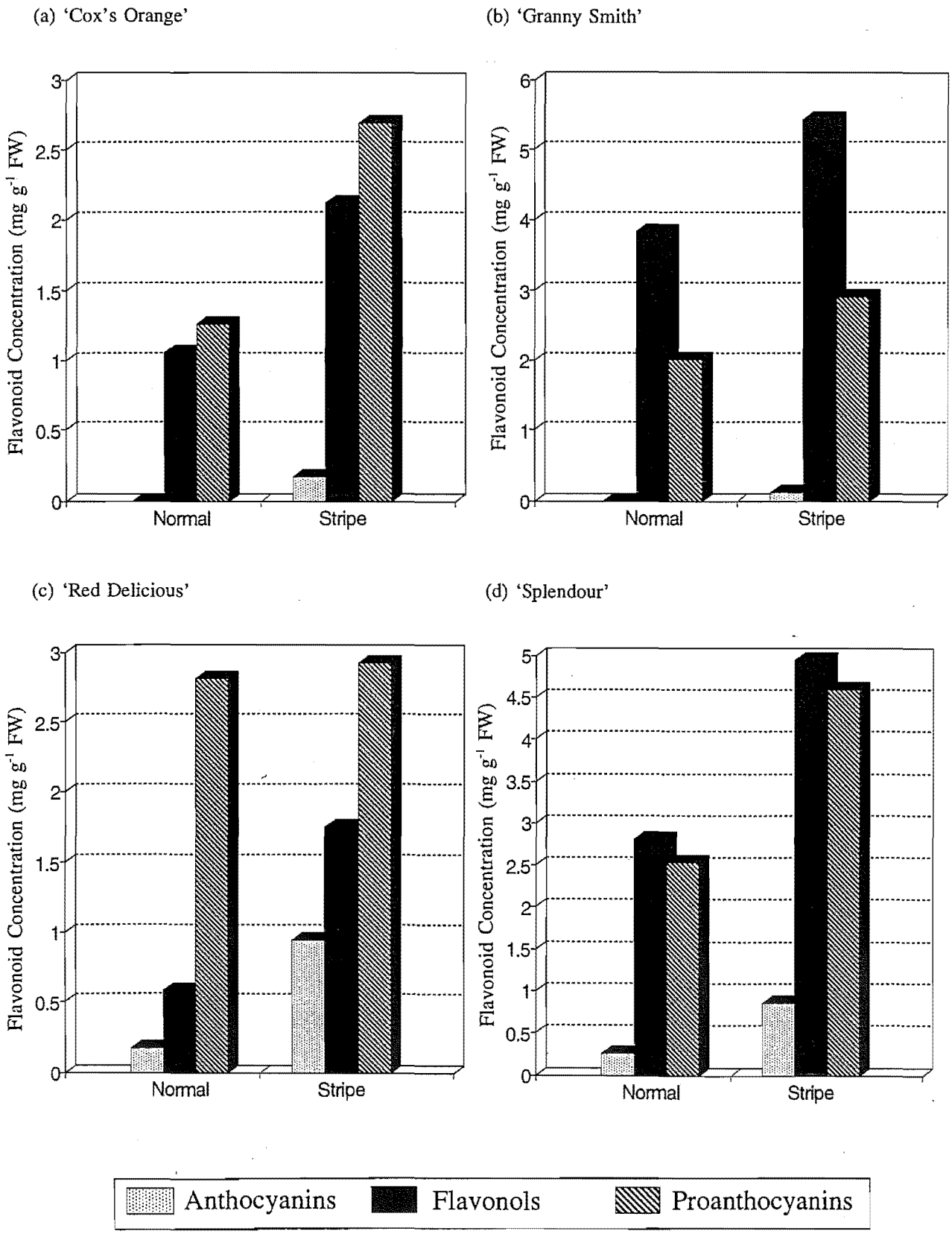


Figure 5.15: Quantification of flavonoid groups in chimeral stripes of four apple cultivars (as determined by HPLC)

Table 5.8: Percentage composition of the flavonoid groups in normal skin and the chimeral stripes of four apple cultivars (as determined by HPLC)

Flavonoid compound	'Cox's Orange'		'Granny Smith'		'Red Delicious'		'Splendour'	
	normal	stripe	normal	stripe	normal	stripe	normal	stripe
1. Flavonols								
Qu-3-galactoside	48.3	48.1	31.1	33.0	31.1	33.4	32.1	33.5
Qu-3-glucoside	7.9	7.4	7.6	6.8	14.0	13.7	11.8	11.9
Qu-3-xyloside	9.1	8.9	13.9	13.2	12.3	11.2	9.2	8.5
Qu-3-arabinofuranoside	20.5	21.4	18.9	18.5	24.7	24.1	18.1	17.6
Qu-3-rhamnoside	8.5	8.7	24.2	24.1	13.6	12.9	22.4	22.0
Other	5.7	5.5	4.3	4.4	4.3	4.7	6.4	6.5
2. Anthocyanin								
Cyanidin-3-galactoside	n.d.*	100	n.d.*	100	100	95.4	94.2	92.9
Other	n.d.*	0	n.d.*	0	0	4.6	5.8	7.1
3. Proanthocyanidins								
Catechin	0.4	0.6	3.3	3.0	0	0	1.1	1.6
Procyanidin B2	34.5	32.8	34.3	36.4	25.9	26.7	27.8	25.2
Epicatechin	36.0	37.5	30.7	32.2	33.4	31.8	37.2	38.3
Procyanidin B5	5.3	4.2	3.1	3.8	6.9	7.5	3.5	3.1
Other	23.8	24.9	28.6	24.6	33.8	34.0	30.4	31.8

* n.d. = not detected

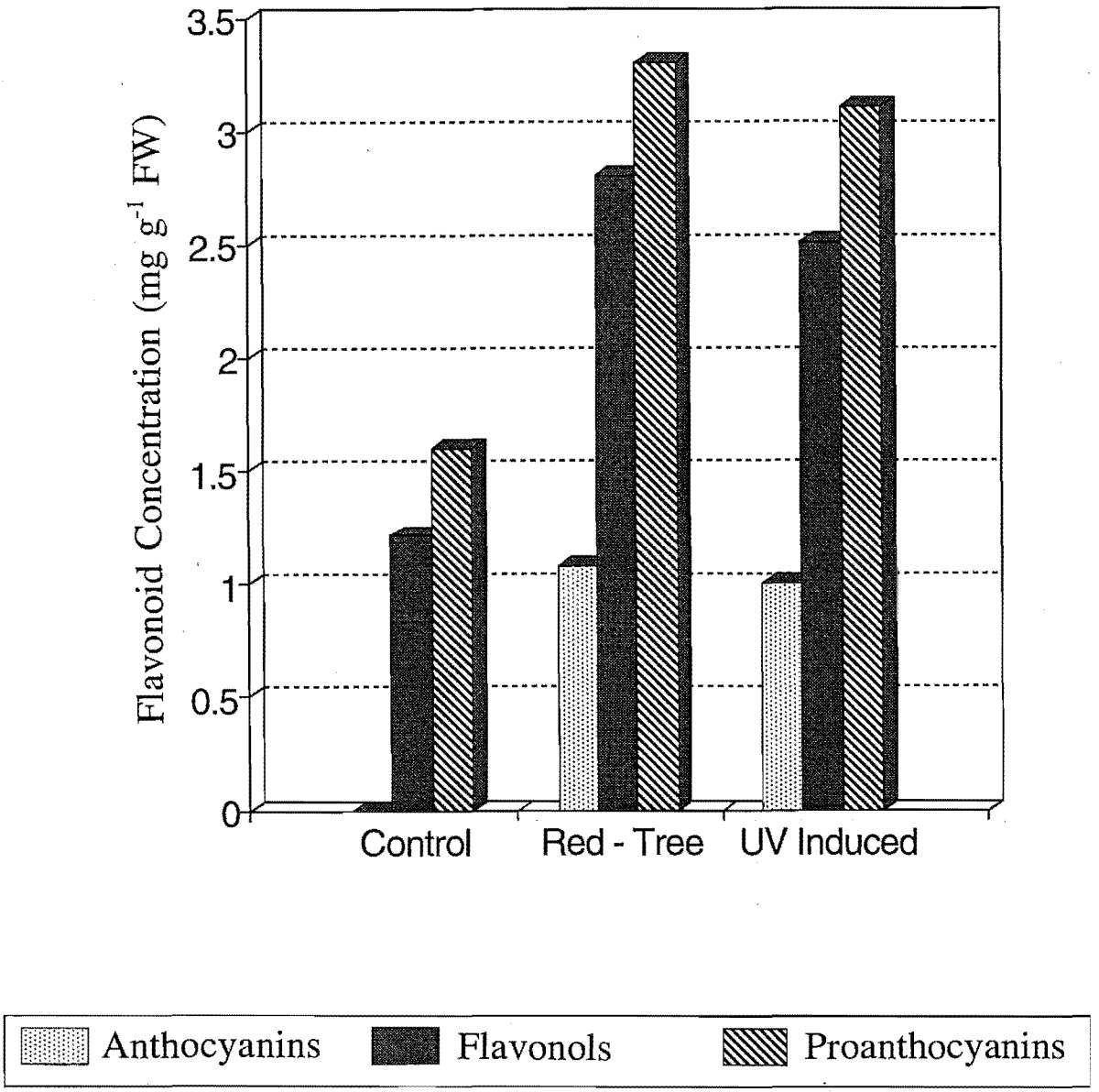


Figure 5.16: Quantification of the flavonoid groups in control, tree-reddened and UV-induced apples (as determined by HPLC)

Table 5.9: Percentage composition of the flavonoid groups in the skin of green control, tree-reddened and red UV induced apples

Flavonoids	Control	Tree-reddened	UV-induced
Anthocyanins			
Cyanidin-3-galactoside	n.d.*	92.9	93.8
Other cyanidin glycosides	n.d.*	7.1	6.2
Flavonols			
Quercetin-3-galactoside	33.2	42.5	40.4
Quercetin-3-glucoside	18.9	16.1	17.3
Quercetin-3-xyloside	13.5	10.8	10.8
Quercetin-3-arabinopyranoside	1.0	2.1	1.7
Quercetin-3-arabinofuranoside	17.1	13.9	14.6
Quercetin-3-rhamnoside	14.2	11.6	12.3
Other	2.1	3.0	2.9
Proanthocyanins			
Catechin	1.1	1.0	0.8
Procyanidin B2	13.0	12.4	11.9
Epicatechin	45.4	47.0	46.0
Gallocatechin	0.3	0.5	0.4
Procyanidin B5	1.8	2.2	1.8
Other Proanthocyanins	38.4	36.9	39.1

* n.d. = not detected

only trace levels to approximately 1 mg g^{-1} FW. However, the increase was not limited to the anthocyanins; and both the flavonols and proanthocyanidins also increasing significantly in concentration. Flavonols rose from 1.2 to 2.5 mg g^{-1} FW and proanthocyanidins from 1.6 to 3.1 mg g^{-1} FW.

These results showed that the compositions of the anthocyanins, flavonols and proanthocyanidins were very similar in the three apple samples, with no significant differences in the compounds present apart from the lack of anthocyanins in the control tissue (Table 5.9). The percentage composition of the proanthocyanidins was similar in all tissues and the flavonols were the same for the red apples from the tree and UV induction. The flavonols were similar except that the two groups of red apples differed from the green control in having a slightly higher percentage of quercetin-3-galactoside.

5.3.4 Discussion

Analysis of the flavonoids in chimeral stripes

It is not really surprising that the increase in flavonoids in the chimeral stripes is not entirely restricted to the anthocyanins due to the close biosynthetic relationship between the anthocyanins, flavonols and proanthocyanidins. However, in 'Red Delicious' there was only a small increase in the proanthocyanidins, which might indicate that a threshold has been reached for this cultivar in the amount of proanthocyanidin that can be produced. This could be due to enzyme activity or the control of proanthocyanidin biosynthesis via a UV threshold limit as has been shown to regulate anthocyanins (Proctor, 1974). The increase in total flavonoids was not due to new compounds, or just restricted to a few individual compounds, but was a general increase indicating an increase in all the enzyme activities rather than a change in glycosylation or an increase in a specific enzyme such as flavonol synthase.

These results would indicate that the mutation commonly occurs in the control mechanism for the whole pathway and a resultant turning on of anthocyanin biosynthesis via "anthocyanin synthase" rather than just an alteration in this factor alone. This mutation could possibly be in the gene(s) involved with the light mediated control of flavonoid and therefore anthocyanin biosynthesis.

UV induction of flavonoids

Results obtained here for the anthocyanins are similar to those obtained previously for apples (Proctor, 1974; Arakawa *et al.*, 1986). However, changes in flavonols and proathocyanidins have not been reported before for apples although they have been studied in some other plants. Similar results have been observed with mustard (*Sinapis alba* L.) cotyledons where anthocyanins and flavonols were shown to respond differently to light although the relative action was the same in both cases (Brödenfeldt & Mohr, 1988). Although both flavonoids increased on light induction but anthocyanin biosynthesis was more responsive showing a swifter response but reaching a plateau earlier. Since the time frames of anthocyanin and flavonol biosynthesis were not examined here it could not be determined if there was a similar response in apples.

These results suggest that, as well as anthocyanin production via "anthocyanin synthase", being turned on by the light treatment there was an increase in the flow of metabolites through the whole flavonoid pathway. This probably stems from an increase in PAL activity which is known to be stimulated by light (Camm & Towers, 1977; Faragher & Chalmers, 1977; Tan, 1979; Jones, 1984) but the other enzymes in the pathway must also be activated, if they are not already, or there must be increased activity. The increase in the relative proportion of quercetin-3-galactoside may have been due to a general increase in galactosyltransferase activity with the induction of anthocyanin biosynthesis. Another explanation for the increase is the induction of a specific cyanidin galactosyltransferase (as part of the "anthocyanin synthase" complex) which can also use quercetin as a substrate.

It has been noted that the proanthocyanidin pathways are entirely constitutive (Stafford, 1990) yet

an increase in proanthocyanidins was noted after UV induction. However, since the activity of the specific enzymes was not measured, the increase may have been entirely due to an increase in intermediates and sufficient enzyme activity at normal levels to accommodate the increased synthesis. There may not have been *de novo* synthesis of the enzyme(s) in the proanthocyanidin pathway. Although flavonols have been reported to be induced by a number of factors (Stafford, 1990) without a study of induction of flavonol synthase it cannot be determined if *de novo* synthesis of this enzyme occurred. The increase in quercetin glycosides may have been due to an increase in the flavonoid precursors due to higher PAL activity since flavonol synthase is presumably already present in non-induced tissues.

In addition to the changes in the flavonoids PAL and CHI activity were measured after UV irradiation of the 'Royal Gala' apples (Dong *et al.*, 1994). PAL activity increased 30-fold after three days irradiation with a steady increase over time. For CHI there was only a four-fold increase which all took place in the first 24 hours. Thus, irradiation of Royal Gala resulted in increased enzyme activity which preceded the increase in anthocyanin concentration. Similar patterns have also been observed for PAL in apple skin by other workers (Faragher & Chalmers, 1977). An increase in PAL is a prerequisite for biosynthesis of anthocyanins and other flavonoids, to generate the necessary precursors. It would appear that no pools of existing flavonoids are available for use for anthocyanin biosynthesis and new precursors must be generated. PAL and CHI were stimulated in a coordinate manner and the coordinate expression of many of the flavonoid genes has been well documented in other plant systems (van Tunen *et al.*, 1988; Martin *et al.*, 1991; Tonelli *et al.*, 1991). In mustard PAL and CHS were both shown to increase but were not the rate-limiting enzymes, particularly in the case of the anthocyanins (Brödenfeldt & Mohr, 1988). It also seems that in apple skin neither PAL nor CHI are the rate-limiting enzymes since their activities would have permitted greater flavonoid accumulation than was observed. Thus, as discussed earlier, the rate-limiting step may be another enzyme or the availability or precursors.

It was also determined that the increase in PAL and CHI was due to *de novo* synthesis of the enzymes and not control at other levels (Dong *et al.*, 1994). Thus, increased enzyme activity, and hence flavonoid accumulation, was a consequence of induction at the level of transcription and not the result of enzyme activation/inactivation.

Control of flavonoid biosynthesis

These results suggest that for flavonoid biosynthesis in apples there is coordinate regulation of the enzymes, including PAL, under UV induction as well as developmental regulation. Activation of "anthocyanin synthase" was accompanied by an increase in other flavonoid enzymes which may be due to common control mechanisms. Many previous studies have concluded that anthocyanin accumulation is mediated by the effects of various factors, such as light, on the level of PAL activity (Faragher, 1983). However these studies would indicate that it is not PAL alone but a controlling factor influencing the

induction of activity of the enzymes of the whole flavonoid pathway. PAL was not the limiting factor in the level of flavonoid accumulation during UV induction and as with endogenous regulation may have been limited by substrate availability or CHS activity.

5.4 Conclusions

The highest levels of enzyme activity (PAL, CHI, and GT) were observed in immature fruit, these dropped during growth and then rose again on ripening, although not to the original levels. Similar changes in the three enzymes were observed in the red ('Splendour') and non-red ('Granny Smith') fruit but the levels of activity were different. Differences in accumulation rates of flavonoids in 'Granny Smith' and 'Splendour' were a function of levels of enzyme activity. The pattern of flavonoid glycosides present in apples was a result of the substrate specificity or the levels of the glycosyltransferases. Changes in CHI and GT activity correlated with changes in flavonoid concentration throughout development indicating their intimate involvement in flavonoid biosynthesis. PAL activity correlated only with the increase in flavonoids, and particularly anthocyanins, on ripening indicating the involvement of PAL in the biosynthesis of other compounds during early development. PAL-IS did not appear to have a significant effect in controlling the changes occurring in flavonoid biosynthesis during the development of apples.

There was coordinate regulation of the flavonoid enzymes, including PAL, during developmental regulation and UV induction of flavonoid biosynthesis. PAL activity is a prerequisite for anthocyanin biosynthesis, and also other flavonoid groups. However the presence of PAL activity does not ensure anthocyanin biosynthesis which must also involve the induction of "anthocyanin synthase". Induction of this appears to require an increase in the activity of PAL, and other flavonoid enzymes in order to provide the necessary intermediates which are not pooled in apples. The changes in flavonoid levels during development were regulated by the control of activity of the enzymes of flavonoid synthesis from PAL onwards. PAL activity was the lowest of the three enzymes yet at all stages of development the theoretical amount of flavonoids that could be produced from this activity exceeding the actual flavonoids accumulated. Thus PAL, CHI or GT did not seem to be rate-limiting in flavonoid biosynthesis and it was more likely to be the availability of phenylalanine or the level of CHS activity.

UV light stimulated not only anthocyanin biosynthesis but also flavonols and proanthocyanins and induced activity of PAL, CHI and presumably "anthocyanin synthase" plus the rest of flavonoid enzymes. In apples there appears to be a series of parallel pathways to each of the flavonoid groups which are controlled by similar mechanisms although this was not determined conclusively.

Section 6 - CONCLUDING DISCUSSION

An understanding of the mechanisms involved in the production of apple skin colour is of scientific interest and the modification of colour by genetic manipulation has potential economic value (Lancaster, 1992). Progress has been made in understanding the biochemical basis of apple fruit skin colour. Beyond an understanding of the biochemical pathway, an understanding of the genes encoding pathway enzymes and/or regulating the expression of the pathway enzymes is essential before genetic manipulation is attempted (Gutterson, 1993). Some potential research areas in the molecular regulation of apple colour have been discussed by Lancaster (1992) and these include determining the degree of homology between genes involved in colour in apples and flowers. Probes for CHS, CHI and DHFR derived from flowers have been used to identify comparable genes in other plants and could be used to identify and clone these genes in apples, providing enough homology exists. Another area worthy of investigation is the changes occurring in mRNA levels, of flavonoid enzymes (e.g. PAL, CHS, CHI and DHFR) during light-induced reddening and determination of the differences between cultivars in their degree of response to UV light. These studies are now being undertaken (Dr. Arend Kootstra, pers. comm.) and an increases in synthesis of PAL and CHI mRNA on UV induction have been noted (Dong *et al.*, 1994).

Investigation of the genes involved in the conversion of leucocyanidin to cyanidin glycosides ("anthocyanidin synthase") is required since little is known about the enzyme(s) involved in this step. Subtractive hybridization of cDNA clones from green and red apple skin could be used to isolate the genes transcribed during reddening. However, since the enzymes of flavonoid biosynthesis appear to be coordinately regulated a number of genes may be transcribed and it may be difficult to determine which ones are specifically involved in the reddening step. *Agrobacterium*-mediated transformation of apples has been reported (James *et al.*, 1989) which opens up the possibility of inserting new genes for the flavonoid pathway into apples. However, before genetic manipulation of apples can be attempted the genes responsible for colour production must be isolated. It has been seen that fruit colour is influenced in a manifold manner and all three pigment groups (carotenoids, chlorophyll and flavonoids) are important and thus modification of the colour trait will require manipulation of the production of several biochemicals and a number of different genes. Flower colour in *Petunia* is estimated to be affected by at least 32 genes (Forkmann, 1991) and a similarly large number of genes may also be expected to be present in apples.

There are three general approaches to controlling colour in plants involving the manipulation of the structural genes: (1) synthesis of a new end product by insertion of genes encoding for a new or missing enzyme of the pathway; (2) reduction in synthesis of an end product which can be achieved by reduction of gene expression through either antisense genes (Ecker & Davis, 1986; Sandler *et al.*, 1988) or co-suppression strategies (Napoli *et al.*, 1990; van der Krol *et al.*, 1990); (3) increasing the concentration of end products by increasing the rate-limiting step(s) (Gutterson, 1993). Manipulation of regulatory genes

may also enable the control of fruit colour by either increasing or decreasing the amount of pigment synthesised. In recent years progress has been made on the genetic manipulation of flower colour but little has been done on fruit, partly due to a lack of understanding of the biochemistry of fruit colour. Now that some knowledge of the biochemistry has been attained the genes responsible for colour can be examined in terms of their potential for use in manipulation of fruit colour. With regard to the flavonoids Forkmann (1991) listed the genes and factors responsible for influencing flower colour:

1) Genes responsible for single steps of the biosynthesis of the various flavonoid classes.

2) Genes, which are responsible for modification of flavonoids.

3) Regulatory genes, which switch on or off the whole pathway or parts of the pathway.

4) Genes and factors influencing flavonoid concentration by: (a) enhancing or reducing pigment synthesis; (b) preventing pigment accumulation; (c) leading to enzymatic or chemical degradation (fading, bleaching)

5) Genes and factors responsible for flower colour pattern by: (a) differential production and/or accumulation of pigments in different areas of the petal; (b) formation of flower chimeras; (c) unstable gene expression due to transposable elements

6) Genes and factors, which influence flower colour by: (a) copigmentation; (b) the interaction of flavonoids with metal ions; (c) the pH of the vacuole

7) Genes controlling morphological characteristics of the flowers such as hairs, papillae, shape and distribution of pigmented cell and type of cuticle.

Some of these genes and factors that are important in influencing colour in flowers do not have a significant effect on fruit colour. With such a number of genes involved in flavonoid biosynthesis the need for a understanding of the biochemistry and the determination of the important and limiting factors on colour in the target plant is apparent. Knowledge of these will allow a directed approach to the manipulation of apple colour rather than a 'hit and miss' approach. The goals of manipulation must also be decided since, although novel colours are desired in flowers and a major emphasis is on the production of blue flowers, this is probably not important in apples. The key target is in the production of evenly coloured red fruit independent of factors such as position in the tree and environmental influences such as light and temperature. Ultimately the goals for genetic manipulation of apples will be market driven by consumer preferences which change periodically and therefore a full understanding of all aspects of colour is required so that these fickle preferences can be met. The various genes and factors outlined above need to be examined in terms of their potential for use in the genetic manipulation of apple fruit colour and the areas requiring further investigation determined.

A number of genes for the individual steps of flavonoid biosynthesis have now been cloned and characterised from some ornamental plants; these include PAL, CHS, CHI, flavanone 3-hydroxylase, DHFR and GT (Forkmann, 1991; Lancaster, 1992; van der Meer *et al.*, 1993). All apples produce all end products so it is not a matter of inserting a gene for a missing step in the pathway. If the desire is to increase red colour then this may be achieved by increasing total flavonoid biosynthesis. One way to do

this is to determine the rate-limiting step(s) and increase it to match the level of input of precursors and the activity of other enzymes. As determined in this study PAL, CHI or GT do not appear to be rate-limiting in apples. However, the presence of isozymes of PAL must also be considered and although total PAL activity is not limiting it is possible that the activity of a flavonoid specific isozyme of PAL is limiting. Further investigation is required firstly to determine if isozymes are present and if this is a limiting step. If the pool of phenylalanine is limiting ways need to be found to increase this, possibly by diverting the flow from some other secondary compound by using antisense to an appropriate gene. In some cases it may also be necessary to increase PAL activity as this may become limiting once flavonoid biosynthesis is increased. CHS may be the critical step and further enzyme studies are required to determine if it is. The genes encoding for CHS have been isolated from a number of plants including *Petunia*, parsley, pea and soybean (Hermann *et al.*, 1988; Koes *et al.*, 1989; Wingender *et al.*, 1989; Harker *et al.*, 1990). This enzyme is encoded by multigene families and the individual CHS genes vary in their expression and response to different environmental signals (Lancaster, 1992). "Anthocyanin synthase" may also be rate-limiting in anthocyanidin production but little is known about this at present and further work is required to characterise this. Once the rate-limiting step(s) is determined then a higher expressing promoter for that gene may be inserted hopefully to increase enzyme activity. Another possibility is to add additional copies of the genes for these enzymes that differ in their response to the various factors. This type of approach has been used in *Petunia* but after transformation with additional sense gene constructs for CHS and DHFR suppression of both the endogenous gene and foreign genes were observed leading to patterned or white flowers (Napoli *et al.*, 1990; van der Krol *et al.*, 1990). Similar effects have since been observed in other plants but the mechanism of this "sense" inhibition is not yet understood and thus the successful application of this technique is uncertain (Forkmann, 1991). Manipulation of structural genes for individual steps alone may not be successful or increase biosynthesis significantly and regulatory genes may also need to be manipulated.

Alteration of flower colour may be achieved by changing the pattern of flavonoids accumulated by insertion of genes responsible for flavonoid modification or blocking of existing genes. These genes include those responsible for the hydroxylation pattern, glycosylation, acylation and methylation of the flavonoids. Generally there is a correlation between colour and the anthocyanidin type (based on hydroxylation pattern) with pelargonidin (3′-OH) producing orange to pink and red shades, cyanidin (3′,4′-OH) giving magenta and crimson, and delphinidin (3′,4′,5′-OH) being the source of purple to blue colours. Apples contain predominantly 3′,4′-hydroxylated anthocyanins and 3′- or 3′,4′,5′-anthocyanins are totally absent. Although only cyanidin is found in apples the range of red colours observed already encompasses these other colours to some extent and ranges from orange to dark red-purple. Thus, the effect of alteration of hydroxylation pattern on the perceived colour is uncertain and it may not be possible to achieve desired colours without changing other factors such as background colour. The hydroxylation pattern may be changed in two ways. Using antisense genes it may be possible to block 3′-hydroxylase gene expression and allow the fruit to accumulate kaempferol and pelargonidin. Alternatively the gene(s)

for 3′,5′-hydroxylase could be inserted to give myricetin and delphinidin derivatives. However, such simple modification may not be possible to achieve. It is possible that apples may have the gene(s) responsible already but the organization into multienzyme complexes or the substrate specificity of some enzymes means that these compounds are not produced. Colours may also be 'muddied' by the presence of background chlorophylls and carotenoids and thus the expected colours may not be produced. This type of manipulation is generally used to attempt to produce colours not usually observed in the target plant, for example blue roses, and thus is probably not a major consideration in the manipulation of apple colour.

Another avenue for manipulation is alteration of the glycosylation pattern of the anthocyanins and/or other flavonoids. All apples showed similar glycosylation patterns of the anthocyanins as well as the flavonols however alteration of this pattern may have limited effect on fruit colour. The nature of the sugar has no influence on the colour (Gross, 1987) but the position of substitution may produce some modification. In apples the flavonoids are all substituted at the 3-position but an additional sugar at the 5-position decreases the absorption at 440 nm (Mazza & Miniati, 1993) and occasionally has been shown to produce more intense colour in some flowers (Forkmann, 1991). A more marked effect has been noted with glycosylation in the 7-position but this is rare. Other modifications of flavonoids may also be possible if methylation and acylation do not occur in apples. Generally acylation of anthocyanin glycosides with aromatic or aliphatic acids does not have a pronounced effect on colour (Forkmann, 1991). However, acylation may sometimes be important for the stabilization of anthocyanins (Brouillard, 1988). Although stability is not a problem in apples if other factors are altered it may become an issue and stabilization may be achieved by insertion of genes for acylation. Methylation has been noted to have a negligible effect (Gross, 1987) although occasionally a small reddening effect has been observed (Forkmann, 1991). Thus, alteration of these types of modifying factors is not worthwhile in apples. Even if the genes responsible for these modifications were inserted into apples alteration of the flavonoid pattern may not be achieved for a number or reasons. It may not be the genes themselves that are responsible for the pattern of flavonoids seen but the control of them that is important. In many cases these genes may actually be present but not expressed, or the enzymes may not be as active or may have lower substrate specificity. The acylated or methylated compounds may not be glycosylated and thus not accumulated due to their instability.

The key areas in the manipulation of apple colour probably lie in the regulatory genes and genes and/or factors controlling flavonoid concentration and these should be the major target of future research. The genes involved in the flavonoid biosynthetic pathway show a complex regulation with respect to the environment, developmental stage and tissue type. Regulation of gene expression is achieved by multiple genes and different promoters or by multiple promoters on the same coding region (Lancaster, 1992). Many regulatory genes are involved and some of these have been cloned and characterised, largely from *Zea mays* (Forkmann, 1991) and more recently from flowers such as *Petunia* (Gerats *et al.*, 1990, van der Meer *et al.*, 1993). These genes may either be directly involved in the regulation of expression of structural genes or have secondary effects on flower colour such as modulation of pH. Since the flavonoid

pathway appears to be coordinately regulated it may be possible to identify genes controlling total synthesis of the flavonoids and alter their expression. For example the *R* gene of maize has been shown to induce all structural genes required for anthocyanin biosynthesis when introduced transiently (Gutterson, 1993). Similar genes may be present in apples. Another area to target is the light regulation of anthocyanin biosynthesis. Different varieties have different energy requirements for anthocyanin biosynthesis (Proctor, 1974). An understanding of this may allow manipulation of the controlling gene(s) involved and thus allow poorly coloured varieties to redden by lowering the energy requirement, or removing it altogether so that even apples receiving little light in the centre of the tree will redden. There has been some work on the regulation of various genes encoding for individual enzymes of the flavonoid pathway. In parsley one CHS gene has been shown to be present with a complex regulation pattern controlled by one promoter with two light-responsive *cis*-acting DNA sequences (Schulze-Lefert *et al.*, 1989). These sequences show homology to light-responsive sequences of genes involved in photosynthesis. Alteration of these promoters or regulatory genes may be able to alter the response of these genes to environmental stimuli such as light may alter flavonoid accumulation. Investigation of the regulatory genes or sequences present in apples is required and alteration of these may be an approach to use for the manipulation of fruit colour.

An important regulatory step in apples was seen to be the control of the "anthocyanin synthase" step. In non-red cultivars blushing is a major reason for rejection of large quantities of fruit. Thus, it may be desirable to block anthocyanin accumulation in these cultivars under all conditions, to give totally green or yellow coloured fruit. This could possibly be achieved through the use of antisense genes to "anthocyanin synthase" but since neither the enzymes(s) or gene(s) have been characterised this is not yet achievable. It may be possible to totally block flavonoid production using antisense genes of an early flavonoid pathway enzyme such as CHS. Antisense expression of CHS in *Petunia* flowers resulted in reduction of CHS activity and pale or white flowers (van der Krol *et al.*, 1988). Apart from the antisense technique the formation of all flavonoids could be prevented by competition with CHS for substrates (Forkmann, 1991). Resveratrol synthase competes with CHS for the substrates 4-coumaroyl-CoA and malonyl-CoA and the gene for this enzyme has been cloned (Schröder *et al.*, 1988). Such a technique may be suitable for the manipulation of flower colour but the implications of such an alteration the composition of an edible plant part are an important consideration. Since quite large quantities of flavonoids are accumulated in the plant similarly large quantities of the alternate product may also be expected.

In some red apple cultivars that redden poorly anthocyanin production may need to be maximised. Anthocyanins are developmentally regulated generally being 'switched on' during the ripening process, which thus limits their accumulation to a period of a few weeks. If this could be triggered earlier then accumulation would take place over a longer period enhancing red colouration. There is a need to find the trigger mechanism for the anthocyanin increase associated with ripening. These changes may be associated with the climacteric and it may not be possible to alter these without altering the ripening of

the fruit. Further research is required on the regulation of the "anthocyanin synthase" step to shed further light on how this may be increased to allow greater anthocyanin accumulation. Switching off flavonol and/or proanthocyanidin production via antisense genes may allow greater anthocyanin production but it will also depend on the activity of "anthocyanin synthase" and possibly the activity of isozymes of earlier enzymes in the flavonoid pathway. However, these flavonoids may have a critical role in stabilising anthocyanins through copigmentation and if they are not present the anthocyanins may not stay in a coloured state.

Another group of regulatory genes or factors are those that have a secondary influence on colour by affecting pH or copigmentation. Genes controlling the pH of the vacuole have been identified in *Petunia* and *Primula*, and those involved in the production of copigments have been found in *Cyclamen* and *Primula* flowers (Forkmann, 1991). As we have seen copigmentation was not a factor responsible for different shades of red in apples. However, it must be taken into consideration in the manipulation of the flavonoid biosynthetic pathway. If anthocyanin biosynthesis is increased it may result in decreased flavonol and proanthocyanidin biosynthesis and thus copigmentation may not occur. This may result in a change or colour due to a shift in the λ_{max} back to 523 nm (that of cyanidin-3-galactoside in aqueous pH 3.0 solution). However since the pH of apple skin is closer to 4.0 the loss of stabilization by copigments may result in a loss of colour due to the instability of the anthocyanin which would change to the colourless pseudobase. Work by Lancaster *et al.* (1994) showed the pH of apple cells to be about 3.6 to 4.0 and the indications were that the pH between cells was uniform. Apple anthocyanin extracts proved to be stable over quite a wide pH range and, probably due to the copigmentation, indicating the pH does not have as great an effect as it does in many flowers. Thus, the ability to control vacuolar pH may not result in any significant colour change in apple skin.

The turnover and degradation of anthocyanin was not investigated in this study and it may be important to have some understanding of this. If degrading enzymes are present it may be possible to improve pigmentation by decreasing the action of these by the use of antisense to the genes encoding for them. The presence of such enzymes may also limit the amount of anthocyanin that may be present and thus changing a controlling factor to increase anthocyanin production may have no nett effect. In *Sinapis alba* it was noted that the turnover rate increased with increasing anthocyanin synthesis (Zenner & Bopp, 1987) and if a similar situation exists in apples then increasing anthocyanin synthesis may not result in as high levels as expected due to higher turnover rates.

The pattern of colouring is not as important in fruit as it is in flowers. However, there is some preference for the striping seen in varieties such as Gala. Thus an understanding of the mechanism of this and the ability to transfer this to any variety may be useful and is worth further study.

The final group of factors, those influencing morphological characteristics are not as important for fruit as they are for flowers. Generally in apples anthocyanins were only present in a single layer although in dark red cultivars several coloured layers were present (Lancaster *et al.*, 1994). Thus, if the number of layers that the anthocyanins are produced in can be controlled then it may be possible to control the

intensity of the red colour. Control of vacuolar size may also have implications for reddening and in cultivars where vacuoles are small it may be necessary to determine if this can be increased to improve the potential for reddening. The presence of waxy cuticle layers and fruit gloss may be other factors to consider. These were not examined in this study but they may have some influence on our perception of colour, by having a light scattering effect, and these may need to be manipulated in conjunction with other factors.

There are also other factors besides those involving the flavonoids. Another extremely important factor, particularly for fruit colour such as in apples, is the importance of the background colour due to the carotenoids and chlorophyll and these have a significant effect in modifying the appearance of the fruit. Thus, modifying the flavonoids (anthocyanin) may not have the desired effect unless the background colour is very pale (low in carotenoids and chlorophyll). Therefore, the manipulation of chlorophyll and carotenoid biosynthesis/degradation must be considered. Bird *et al.* (1991) used antisense genes to block carotenoid biosynthesis in tomato fruit. A large reduction in carotenoids was observed in the fruit but not the vegetative tissue. This is important and in apples, if carotenoid biosynthesis is to be reduced, it will be important to only block it only in the fruit or even just the epidermal tissue while maintaining normal levels in the leaves. Thus genes will have to be found that program that pathway in the specific tissue.

A further area requiring study is the non-uniform distribution of anthocyanin between apple fruit epidermal cells observed by Lancaster *et al.* (1994). Thus, it will be important to determine what is controlling these differences in levels and whether differences occur just in the anthocyanins or also other flavonoids. One form of control might be at the level of organisation into multienzyme complexes with small changes having significant effects on the flavonoid levels. The organisation of the flavonoid pathway and the existence of multienzyme complexes in apples needs to be determined. If such complexes exist then the control and regulatory factors influencing these may be critical factors and the ability to manipulate these may be useful.

When considering the manipulation of fruit colour there are a number of other factors that must be taken into consideration. In the manipulation of flower colour problems have arisen with variegation and unstable expression of introduced genes. Therefore there is a need to elucidate the mechanism(s) which will guarantee stable expression and inheritance of these introduced genes (Forkmann, 1991). Work is currently being undertaken with flowers to understand this and it will contribute to the goal of manipulating fruit colour. Another important consideration is that if genes are inserted then it is important that they are expressed only in the epidermal layers and tissue-specific promoters or genes need to be identified. Genes have been identified that control anthocyanin distribution to different parts of the flower (Forkmann, 1991) and also the tissue distribution in maize (van der Meer *et al.*, 1993) but there are no reports of epidermal specific ones.

The functions of the flavonoids in the plant must not be ignored and although they are secondary products they may play important roles and decreasing flavonoid levels may have significant consequences on plant health. The major function of flavonoids as attractants for seed dispersal is not important in a

commercial orchard but some other functions are. If they have a role as UV protectants decreasing the levels could cause harmful effects such as DNA and RNA damage which could result in changes in sensory characteristics or alteration of ripening or development of the fruit. Another consideration is that high levels of flavonoids in fruits have been purported to be beneficial to human health. Some flavonoids, including quercetin, were originally classed as vitamin P and were claimed to decrease the permeability of blood vessels. These compounds are no longer classed as vitamins due to the lack of evidence that they are essential in the diet. More recently flavonoids, including quercetin, have been suggested as having a role in reducing death from coronary heart disease, with one of the major dietary sources being apples (Hertog *et al.*, 1993). The mechanism of action of these compounds was not discussed and more research is required to substantiate these claims.

The successful manipulation of apple colour to achieve desired fruit colours still lies some way off. A prerequisite for successful genetic manipulation is a detailed knowledge of all parameters involved in the expression of the respective pigments. Progress has now been made into the understanding of the biochemistry of apple colour which is the first step in the pathway. Now there is a need to start understanding the genetic control of pigment biosynthesis to give further insight into control mechanisms for colour. Knowledge of the general function and interaction of genes, enzymes and further factors involved in the pigment pathway is also required. Some understanding of these is now being gained from the manipulation of flower colour. Modification of apple colour is likely to be a complex matter and may involve the manipulation of a number of different genes involved in the control and biosynthesis of the pigment groups but through an understanding of the factors involved it may be achievable.

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APPENDIX A

Source and description of apple genotypes and species

Name[a]	Source[b]	Parentage	Description
1/1	LUO	'Splendour' x 'Cox's Orange'	Dark red, slightly bluish Yellow background Light yellow flesh
1/21	LUO	'Splendour' x 'Ballarat'	Mid red Yellow background Pale yellow flesh
3/11	LUO	'Splendour' x 'Ballarat'	Pink-red Light yellow-green background
3/16	HNRO	Sport of 'Gala'	Pink-red striping Yellow background
3827	HNRO	'Golden Delicious' x 'Red Dougherty'	Mid red over ½ fruit, some light red blush Yellow background
4692	HRNO	'Golden Delicious' x 'Red Dougherty'	Pink-red blush appearing bronzy Green background Greenish flesh
4926	HRNO	'Golden Delicious' x 'Red Dougherty'	Bright red over most of fruit Bright yellow background Yellow flesh
4/1	HNRO	Sport of 'Gala'	Mid red stripes Bright yellow background Yellow flesh

Name[a]	Source[b]	Parentage	Description
4/7	HNRO	Sport of 'Gala'	Mid red striping Yellow background Light yellow flesh
4/17	LUO	'Kent' - 'Cox' derivative	'Gala'-type striping mid-red Bright green-bronzy background
4 last	LUO	'Cox's Orange' derivative	Mid red stripes Orange-bronze background Yellow flesh
666	HNRO		Light red blush over ½ of fruit Very yellow background
92 Just	HNRO	Unknown seedling	Mid to dark red over most of fruit, mottled Yellow background Yellow flesh
'Braeburn'	COS	Chance seedling from Nelson, N.Z.	Bronze-red blush over ½ of fruit Green background Light greenish-yellow flesh
'Cox's Orange Pippin'	LUO	Chance seedling UK	Dark red striping on part of fruit Yellow background and flesh
'Fuji'[1] 'Fuji'[2]	HNRO COS	'Ralls Janet' x 'Delicious'	Mid red blushing over ½ of fruit Green background Pale yellow to green flesh
'Gala'		N.Z. breeding programme	Mid red striping Yellow background and flesh
'Gavin'	HNRO		Dark red skin over most of apple Green background

Name[a]	Source[b]	Parentage	Description
'Golden Delicious'	LUO	'Grimes Golden' (rep)	Bright yellow, no red at maturity Yellow flesh
'Granny Smith'	HNRO	Chance seedling from Australia	Occasional light red blush Bright green background Light green flesh
GS 109	HNRO	'Gala' x 'Splendour'	Very dark red with bluish tint Yellow background Yellowish flesh
GS 150	HNRO	'Gala' x 'Splendour'	Pinky to cherry red most of fruit Yellow background Slightly yellow flesh
Hyde 5/22	HNRO		Dark to mid red with indistinct striping Yellow background and flesh
Hyde 5/30	HNRO		'Gala'-type with bright red stripes Yellow background Yellowish flesh
'Ikorokavka Alajah'	LUO		Bright pink-red Very pale creamy background White flesh
J997	HNRO	Japanese breeding programme	Pink to mid red striping over ½ fruit Yellow background Yellowish flesh
'Lawfam'	HNRO	'Fameuse' (rep) x 'Lawver'	Very dark red with bluish tint Light green background and flesh

Name[a]	Source[b]	Parentage	Description
'McKenzie'	HNRO	'Golden Delicious' x 'Red Dougherty'	Light red blush (bronzy) over ½ fruit Bright green background Green flesh
'Maling 9'	HNRO	Rootstock	Mottled green and bright yellow, absolutely no red Pale green flesh
Malus micromalus	HNRO	Hybrid crab apple	Red to pink-red over most of fruit Yellow background and flesh
Malus pumila niedzwetzkyana	HNRO		Dark to mid red over most of fruit Yellow background Pink flesh
Malus soulardii	HNRO	Hybrid *M.ioensis* & common apple	Large fruiting crab apple Bright yellow skin, some green Very yellow flesh
Malus sylvestris	HNRO	An ancestor of the domestic apple	Yellow skin with brown russet, no red at all Whitish flesh
'Oregon Red'	HNRO	Sport of 'Red Delicious'	Very dark red with blue-black tint Yellow flesh
'Red Cox'	HNRO	Sport of 'Cox's Orange'	Dark red with bluish tint Yellow background Slightly yellow flesh
'Red Delicious'	LUO	Chance seedling USA	Mid red (this strain not as dark blue-purple as usual) Yellow flesh

Name[a]	Source[b]	Parentage	Description
'Regal Gala'	HNRO	'Gala' sport	Mid to dark red with indistinct striping Yellow background and flesh
'Spartan'	LUO	'Newtown Pippin' x 'McIntosh'	Dark red with bluish tint over entire fruit Very white flesh
'Splendour'	LUO		Mid red over most of fruit Light green to yellow background Pale yellow flesh
'Sturmer'	LUO	'Nonpariel' x 'Ribston Pippin'	Slight pale red blush over ½ fruit Bright green background Greenish flesh

[a] In some cases genotypes are not commercial cultivars and so were assigned numbers

[b] COS - Canterbury Orchard Systems
HNRO - Hortresearch Havelock North Research Orchard
LUO - Lincoln University Orchard

APPENDIX B

Concentrations of flavonoids in apple genotypes and species

Key:

Flavonols

Q-gal	Quercetin-3-galactoside
Q-rut	Quercetin-3-rhamnoglucoside
Q-glu	Quercetin-3-glucoside
Q-xyl	Quercetin-3-xyloside
Q-a(p)	Quercetin-3-arabinopyranoside
Q-a(f)	Quercetin-3-arabinofuranoside
Q-?	Unknown quercetin glycoside
Q-rha	Quercetin-3-rhamnoside
K-gly	Kaempferol glycosides

Anthocyanins

Cy-gal	Cyanidin-3-galactoside
Cy-gly	Other cyanidin glycosides

Proanthocyanidins

Cat	(+)-Catechin
Pro B2	Procyanidin B2
Epi	(-)-Epicatechin
Gallo	(+)-Gallocatechin (putative)
Pro B5	Procyanidin B5
Other	Other proanthocyanidins

Apple genotype or species	Flavonol concentration (μg g^{-1})									
	Q-gal	Q-rut	Q-glu	Q-xyl	Q-a(p)	Q-a(f)	Q-?	Q-rha	K-gly	Total
1/1	2470	71	669	584	90	1171	165	830	253	6303
1/21	3055	68	1342	512	93	1280	100	775	176	7401
3/11	1881	90	1685	394	59	789	96	1729	200	6923
3/16	2224	57	424	405	49	694	64	516	54	4487
3827	1487	31	592	385	49	839	61	709	82	4235
4692	1944	45	831	407	65	771	57	700	N.D.	4820
4926	2024	34	1211	485	74	907	68	726	167	5696
4/1	1802	62	446	310	63	644	48	316	N.D.	3691
4/7	1404	84	195	299	40	602	57	279	19	2979
4/17	1324	35	578	281	51	610	54	577	137	3647
4 last	4184	109	1441	1276	173	1453	148	520	255	9559
666	1917	56	922	581	74	982	49	487	41	5109
92 Just	1084	22	230	482	80	562	74	436	278	3248
'Braeburn'	2870	36	532	708	95	1114	52	644	135	6186
'Cox's Orange Pippin'	776	38	99	123	23	292	22	1114	N.D.	1487
'Fuji'[1]	3746	64	1116	926	164	1521	131	932	110	8710
'Fuji'[2]	2813	43	803	701	106	992	66	633	68	6225
'Golden Delicious'	724	33	117	137	13	362	26	314	N.D.	1726

Apple genotype or species	Flavonol concentration (μg g^{-1})									
	Q-gal	**Q-rut**	**Q-glu**	**Q-xyl**	**Q-a(p)**	**Q-a(f)**	**Q-?**	**Q-rha**	**K-gly**	**Total**
'Granny Smith'[3]	1023	84	236	407	29	649	55	745	N.D.	3228
'Granny Smith'[4]	990	17	484	439	41	799	112	633	50	3566
GS 109	1278	109	577	379	81	421	37	268	N.D.	3150
GS 150[3]	2055	42	873	379	96	730	70	783	170	5198
GS 150[4]	2421	45	951	340	86	660	69	885	207	5664
Hyde 5/22	1102	80	243	268	57	536	58	241	N.D.	2585
Hyde 5/30	1778	87	446	308	68	623	42	391	29	3772
'Ikorokavka Alajah'	1496	41	531	508	63	808	58	461	127	4093
J997[3]	1459	86	456	492	98	727	56	447	458	4279
J997[4]	1691	37	605	607	134	970	83	487	670	5284
'Lawfam'[3]	3375	52	1700	970	166	1795	97	1293	496	9944
'Lawfam'[4]	3051	61	2074	936	148	1668	117	1226	865	10146
'McKenzie'	1872	70	602	386	31	491	39	460	12	3963
Maling 9	439	27	221	178	20	449	16	360	29	1739
Malus micromalus	1419	40	404	504	67	969	89	425	N.D.	3917
Malus pumila niedzwetzkyana	986	44	217	155	28	417	25	146	37	2055
Malus soulardii	353	17	112	89	16	212	11	78	14	902
Malus sylvestris	129	5	38	76	7	203	25	33	108	624

Apple genotype or species	Flavonol concentration (μg g^{-1})									
	Q-gal	**Q-rut**	**Q-glu**	**Q-xyl**	**Q-a(p)**	**Q-a(f)**	**Q-?**	**Q-rha**	**K-gly**	**Total**
'Oregon Red'[3]	2336	61	273	844	137	1262	88	801	405	6206
'Oregon Red'[4]	2088	45	264	758	113	1259	146	681	560	5914
'Red Cox'	1293	53	204	205	43	478	43	196	N.D.	2515
'Red Delicious'	995	66	400	338	47	736	86	418	N.D.	3086
'Regal Gala'[3]	1128	28	193	338	55	577	50	390	150	2909
'Regal Gala'[4]	1534	33	279	561	67	779	91	501	244	4089
Seedling Gavin	1916	84	992	558	101	949	63	354	N.D.	5017
'Spartan'	581	55	137	156	43	422	32	149	116	1691
'Splendour'[3]	1827	101	519	390	43	809	51	1066	N.D.	4808
'Splendour'[4]	1805	31	561	416	65	830	103	991	117	4919
'Sturmer'	4088	211	1267	595	71	1229	61	747	66	8255
LSD's 5%	1005	67	532	187	31	289	34	338	100	2319

N.D. Not detected

[1] Sample collected from Havelock North Research Orchard

[2] Sample collected from Canterbury Orchard Systems

[3] Sample collected in 1990

[4] Sample collected in 1991

Apple genotype or species	Anthocyanins (μg g^{-1})			Proanthocyanidin concentration (μg g^{-1})							Phloridzin (μg/g)
	Cy-gal	Cy-gly	Total	Cat	Pro B2	Epi	Gallo	Pro B5	Other	Total	
1/1	1340	53	1393	114	1135	1986	97	192	919	4443	18.03
1/21	226	7	233	115	416	716	52	84	174	1557	23.20
3/11	360	13	373	41	695	752	49	74	303	1914	21.00
3/16	163	16	179								19.19
3827	537	18	555	32	468	612	31	76	552	1771	9.07
4692	128	N.D.	128								0.00
4926	801	34	835	80	683	850	54	100	642	2409	8.27
4/1	431	27	458								0.00
4/7	271	24	295								11.32
4/17	722	38	760	21	700	617	34	97	502	1971	14.27
4 last	71	N.D.	71	31	494	347	21	34	379	1306	30.57
666	83	N.D.	83								0.00
92 Just	1479	103	1582	23	994	954	99	159	624	2853	4.00
'Braeburn'	124	N.D.	124								26.63
'Cox's Orange Pippin'	251	14	265								0.00
'Fuji'[1]	N.D.	N.D.	N.D.								21.82
'Fuji'[2]	82	N.D.	82								9.30
'Golden Delicious'	N.D.	N.D.	N.D.								0.00

Apple genotype or species	Anthocyanins (μg g^{-1})			Proanthocyanidin concentration (μg g^{-1})							Phloridzin (μg/g)
	Cy-gal	Cy-gly	Total	Cat	Pro B2	Epi	Gallo	Pro B5	Other	Total	
'Granny Smith'[3]	N.D.	N.D.	N.D.								0.00
'Granny Smith'[4]	N.D.	N.D.	N.D.	66	685	638	16	60	548	2013	17.40
GS 109	1206	130	1336								0.00
GS 150[3]	904	75	979								0.00
GS 150[4]	947	38	985	92	725	995	50	87	860	2809	11.50
Hyde 5/22	1677	148	1825								0.00
Hyde 5/30	432	26	458								11.97
'Ikorokavka Alajah'	486	71	557	21	707	557	52	89	418	1844	10.83
J997[3]	576	35	611								12.23
J997[4]	1337	105	1442	54	550	675	21	129	602	2031	60.91
'Lawfam'[3]	2111	242	2353								25.92
'Lawfam'[4]	953	88	1041	80	390	573	76	8	493	1620	37.46
McKenzie	50	N.D.	50								0.00
Maling 9	N.D.	N.D.	N.D.								0.00
Malus micromalus	690	37	727								0.00
Malus pumila niedzwetzkyana	1051	80	1131								0.00
Malus soulardii	13	N.D.	13								0.00
Malus sylvestris	N.D.	N.D.	N.D.								0.00

Apple genotype or species	Anthocyanins (μg g^{-1})			Proanthocyanidin concentration (μg g^{-1})							Phloridzin (μg/g)
	Cy-gal	Cy-gly	Total	Cat	Pro B2	Epi	Gallo	Pro B5	Other	Total	
'Oregon Red'[3]	3013	234	3247								0.00
'Oregon Red'[4]	4139	249	4388	1	1684	2043	238	443	1848	6257	11.05
'Red Cox'	2596	137	2733								0.00
'Red Delicious'	880	57	937								0.00
'Regal Gala'[3]	1905	113	2018								0.00
'Regal Gala'[4]	1945	97	2042	85	649	1135	38	127	939	2973	12.72
Seedling Gavin	1082	99	1181								0.00
'Spartan'	1084	25	1109								0.00
'Splendour'[3]	820	66	886								0.00
'Splendour'[4]	1055	123	1178	53	927	1312	60	98	1109	3559	29.03
'Sturmer'	56	N.D	56								11.07
LSD's 5%	431	71	484	36	238	388	48	62	456	1322	11

N.D. Not detected

[1] Sample collected from Havelock North Research Orchard

[2] Sample collected from Canterbury Orchard Systems

[3] Sample collected in 1990

[4] Sample collected in 1991

APPENDIX C

Percentage composition of flavonoids in apple genotypes and species

Key:

Flavonols

Q-gal	Quercetin-3-galactoside
Q-rut	Quercetin-3-rhamnoglucoside
Q-glu	Quercetin-3-glucoside
Q-xyl	Quercetin-3-xyloside
Q-a(p)	Quercetin-3-arabinopyranoside
Q-a(f)	Quercetin-3-arabinofuranoside
Q-?	Unknown quercetin glycoside
Q-rha	Quercetin-3-rhamnoside
K-gly	Kaempferol glycosides

Anthocyanins

Cy-gal	Cyanidin-3-galactoside
Cy-gly	Other cyanidin glycosides

Proanthocyanidins

Cat	(+)-Catechin
Pro B2	Procyanidin B2
Epi	(-)-Epicatechin
Gallo	(+)-Gallocatechin (putative)
Pro B5	Procyanidin B5
Other	Other proanthocyanidins

Apple genotype or species	Flavonols (%)									
	Q-gal	Q-rut	Q-glu	Q-xyl	Q-a(p)	Q-a(f)	Q-?	Q-rha	K-gly	Total
1/1	39.2	1.1	10.6	9.3	1.4	18.6	2.6	13.2	4.0	100
1/21	41.3	0.9	18.1	6.9	1.2	17.3	1.4	10.5	2.4	100
3/11	27.2	1.3	24.3	5.7	0.8	11.4	1.4	25.0	2.9	100
3/16	49.6	1.3	9.4	9.0	1.1	15.5	1.4	11.5	1.2	100
3827	35.1	0.7	14.0	9.1	1.2	19.8	1.5	16.7	1.9	100
4692	40.3	0.9	17.2	8.5	1.4	16.0	1.2	14.5	0	100
4926	35.6	0.6	21.3	8.5	1.3	15.9	1.2	12.7	2.9	100
4/1	48.8	1.7	12.1	8.4	1.7	17.4	1.3	8.6	0	100
4/7	47.1	2.8	6.6	10.0	1.4	20.2	1.9	9.4	0.6	100
4/17	36.3	1.0	15.9	7.7	1.4	16.7	1.5	15.8	3.7	100
4 last	43.8	1.1	15.1	13.4	1.8	15.2	1.5	5.4	2.7	100
666	37.5	1.1	18.0	11.4	1.5	19.2	1.0	9.5	0.8	100
92 Just	33.4	0.7	7.1	14.8	2.5	17.3	2.3	13.4	8.5	100
'Braeburn'	46.4	0.6	8.6	11.4	1.5	18.0	0.9	10.4	2.2	100
'Cox's Orange Pippin'	52.2	2.5	6.6	8.3	1.5	19.7	1.5	7.7	0	100
'Fuji'[1]	43.0	0.7	12.8	10.6	1.9	17.5	1.5	10.7	1.3	100
'Fuji'[2]	45.2	0.7	12.9	11.3	1.7	15.9	1.0	10.2	1.1	100
'Golden Delicious'	42.0	1.9	6.8	7.9	0.7	21.0	1.5	18.2	0	100

Apple genotype or species	Flavonols (%)									
	Q-gal	**Q-rut**	**Q-glu**	**Q-xyl**	**Q-a(p)**	**Q-a(f)**	**Q-?**	**Q-rha**	**K-gly**	**Total**
'Granny Smith'[3]	31.7	2.6	7.3	12.6	0.9	20.1	1.7	23.1	0	100
'Granny Smith'[4]	27.8	0.5	13.6	12.3	1.1	22.4	3.1	17.8	1.4	100
GS 109	40.6	3.5	18.3	12.0	2.5	13.4	1.2	8.5	0	100
GS 150[3]	39.6	0.8	16.8	7.3	1.8	14.0	1.3	15.1	3.3	100
GS 150[4]	42.8	0.8	16.8	6.0	1.5	11.7	1.2	15.6	3.7	100
Hyde 5/22	42.6	3.1	9.4	10.4	2.2	20.7	2.3	9.3	0	100
Hyde 5/30	47.1	2.3	11.8	8.2	1.8	16.5	1.1	10.4	0.8	100
'Ikorokavka Alajah'	36.6	1.0	13.0	12.4	1.5	19.7	1.4	11.3	3.1	100
J997[3]	34.1	2.0	10.7	11.5	2.3	17.0	1.3	10.4	10.7	100
J997[4]	32.0	0.7	11.4	11.5	2.5	18.4	1.6	9.2	12.7	100
'Lawfam'[3]	33.9	0.5	17.1	9.8	1.7	18.0	1.0	13.0	5.0	100
'Lawfam'[4]	30.1	0.6	20.4	9.2	1.5	16.4	1.2	12.1	8.5	100
'McKenzie'	47.2	1.8	15.2	9.7	0.8	12.4	1.0	11.6	0.3	100
Maling 9	25.2	1.6	12.7	10.2	1.2	25.8	0.9	20.7	1.7	100
Malus micromalus	36.2	1.0	10.3	12.9	1.7	24.7	2.3	10.9	0	100
Malus pumila niedzwetzkyana	48.0	2.1	10.6	7.5	1.3	20.3	1.2	7.1	1.8	100
Malus soulardii	39.1	1.8	12.4	9.8	1.8	23.5	1.3	8.7	1.6	100
Malus sylvestris	20.7	0.8	6.1	12.3	1.1	32.5	4.0	5.2	17.3	100

Apple genotype or species	Flavonols (%)									
	Q-gal	Q-rut	Q-glu	Q-xyl	Q-a(p)	Q-a(f)	Q-?	Q-rha	K-gly	Total
'Oregon Red'[3]	37.6	1.0	4.4	13.6	2.2	20.3	1.4	12.9	6.6	100
'Oregon Red'[4]	35.3	0.7	4.5	12.8	1.9	21.3	2.5	11.5	9.5	100
'Red Cox'	51.4	2.1	8.1	8.2	1.7	19.0	1.7	7.8	0	100
'Red Delicious'	32.3	2.1	13.0	11.0	1.5	23.8	2.8	13.5	0	100
'Regal Gala'[3]	38.8	1.0	6.6	11.6	1.9	19.8	1.7	13.4	5.2	100
'Regal Gala'[4]	37.5	0.8	6.8	13.7	1.6	19.1	2.2	12.3	6.0	100
Seedling Gavin	38.2	1.7	19.8	11.1	2.0	18.9	1.2	7.1	0	100
'Spartan'	34.4	3.2	8.1	9.2	2.6	24.9	1.9	8.8	6.9	100
'Splendour'[3]	38.0	2.1	10.8	8.1	0.9	16.8	1.1	22.2	0	100
'Splendour'[4]	36.7	0.6	11.4	8.4	1.3	16.9	2.1	20.2	2.4	100
'Sturmer'	48.6	2.5	15.3	7.2	0.9	14.9	0.7	9.1	0.8	100
LSD's 5%	5.7	1.9	4.0	1.8	0.5	3.7	1.0	3.9	3.1	-

[1] Sample collected from Havelock North Research Orchard
[2] Sample collected from Canterbury Orchard Systems
[3] Sample collected in 1990
[4] Sample collected in 1991

Apple genotype or species	Anthocyanins (%)			Proanthocyanidins (%)						
	Cy-gal	Cy-gly	Total	Cat	Pro B2	Epi	Gallo	Pro B5	Other	Total
1/1	96.2	3.8	100	2.6	25.5	44.7	2.2	4.3	20.7	100
1/21	97.1	2.9	100	7.4	26.7	46.0	3.3	5.4	11.2	100
3/11	96.5	3.5	100	2.1	36.3	39.3	2.6	3.8	15.9	100
3/16	91.0	9.0	100							
3827	96.8	3.2	100	1.8	26.4	34.5	1.8	4.3	31.2	100
4692	100	0	100							
4926	96.0	4.0	100	3.3	28.4	35.3	2.2	4.2	26.6	100
4/1	94.2	5.8	100							
4/7	91.9	8.1	100							
4/17	95.0	5.0	100	1.1	35.5	31.3	1.7	4.9	25.5	100
4 last	100	0	100	2.4	37.8	26.6	1.6	2.6	29.0	100
666	100	0	100							
92 Just	93.5	6.5	100	0.8	34.8	33.4	3.5	5.6	21.9	100
'Braeburn'	100	0	100							
'Cox's Orange Pippin'	94.8	5.2	100							
'Fuji'[1]	-	-	-							
'Fuji'[2]	100	0	100							
'Golden Delicious'	-	-	-							

Apple genotype or species	Anthocyanins (%)			Proanthocyanidins (%)						
	Cy-gal	Cy-gly	Total	Cat	Pro B2	Epi	Gallo	Pro B5	Other	Total
'Granny Smith'[3]	-	-	-							
'Granny Smith'[4]	-	-	-	3.3	34.0	31.7	0.8	3.0	27.2	100
GS 109	90.3	9.7	100							
GS 150[3]	92.4	7.6	100							
GS 150[4]	96.2	3.8	100	3.3	25.8	35.4	1.8	3.1	30.6	100
Hyde 5/22	91.9	8.1	100							
Hyde 5/30	94.2	5.8	100							
'Ikorokavka Alajah'	87.2	12.8	100	1.1	38.4	30.2	2.8	4.8	22.7	100
J997[3]	94.3	5.7	100							
J997[4]	92.7	7.3	100	2.7	27.1	33.2	1.0	6.3	29.7	100
'Lawfam'[3]	89.7	10.3	100							
'Lawfam'[4]	91.5	8.5	100	4.9	24.1	35.4	4.7	0.5	30.4	100
'McKenzie'	-	-	-							
Maling 9	-	-	-							
Malus micromalus	94.9	5.1	100							
Malus pumila niedzwetzkyana	92.9	7.1	100							
Malus soulardii	100	0	100							
Malus sylvestris	-	-	-							

Apple genotype or species	Anthocyanins (%)			Proanthocyanidins (%)						
	Cy-gal	Cy-gly	Total	Cat	Pro B2	Epi	Gallo	Pro B5	Other	Total
'Oregon Red'[3]	92.8	7.2	100							
'Oregon Red'[4]	94.3	5.7	100	0.0	26.9	32.7	3.8	7.1	29.5	100
'Red Cox'	95.0	5.0	100							
'Red Delicious'	93.9	6.1	100							
'Regal Gala'[3]	94.4	5.6	100							
'Regal Gala'[4]	95.2	4.8	100	2.8	21.8	38.2	1.3	4.3	31.6	100
Seedling Gavin	91.6	8.4	100							
'Spartan'	97.8	2.2	100							
'Splendour'[3]	92.6	7.4	100							
'Splendour'[4]	89.5	10.5	100	1.5	26.0	36.9	1.7	2.7	31.2	100
'Sturmer'	-	-	-							
LSD's 5%	3.5	3.4	-	1.2	9.4	12.5	1.6	1.9	13.4	-

[1] Sample collected from Havelock North Research Orchard
[2] Sample collected from Canterbury Orchard Systems
[3] Sample collected in 1990
[4] Sample collected in 1991

APPENDIX D

Papers published in relation to this thesis

Lancaster, J.E.; Grant, J.E.; Lister, C.E.; Taylor, M.C. (1994). Skin color in apples - influence of copigmentation and plastid pigments on shade and darkness of red colour in five genotypes. *J. Amer. Soc. Hort. Sci.* **119(1)**: 63-69.

Lister, C.E.; Lancaster, J.E.; Sutton, K.; Walker, J.R.L. (1994). Developmental changes in the concentration and composition of flavonoids in skin of a red and a green apple cultivar. *J. Sci. Food Agric.* **64**: 155-161.

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Skin Color in Apples—Influence of Copigmentation and Plastid Pigments on Shade and Darkness of Red Color in Five Genotypes

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Abstract. **The biochemical and cytological mechanisms responsible for the differences in red color quality of apples (*Malus domestica* Borkh.) were investigated. Copigmentation, the increase in absorbance maxima (λ max) from anthocyanin and flavonoid interactions, is known to be a mechanism for producing variation in shade of red in flowers. In intact apple skin cells, the mean λ max was 550 nm, with no significant difference between genotypes. Furthermore, the ratio of flavonols and proanthocyanidins to anthocyanins was similar for all genotypes. Therefore, copigmentation is not a mechanism producing different shades of red in apples. Darkness of red skin was positively related to the proportion of red cells in the skin and the size of the vacuoles containing anthocyanins. Measurements of plastid pigments, chlorophyll, and carotenoids, compared with L*, a*, b* measurements, indicated that the visual blending of plastid pigments and anthocyanins has an important influence on red coloration of apple skin.**

Apple skin color is caused by the pigments chlorophyll and carotenoids located in plastids and the phenolic pigments (anthocyanin, flavonols, and proanthocyanidins) located in the vacuole. The flavonols and proanthocyanidins do not contribute significantly to overall coloration but may be important in enhancing anthocyanin coloration by copigmentation.

Color is traditionally measured by destructive techniques, such as isolation and quantitation of pigments, or by nondestructive measurements based on determination of the characteristics of light, transmitted or reflected, by the fruit. The relationships between pigment composition, color measurement, and perception of color by the eye are complex, and evidence is lacking on the extent to which differences in pigment composition are apparent to the eye as color differences (Knee, 1980). In apples, red coloration is commercially desirable. Variation exists in the shade of red and the pattern of reddening (e.g., solid block or striping). This variation depends on the genotype and on developmental and environmental factors (Lancaster, 1992; Saure, 1990).

In flowers, there has been extensive work on the relationship between petal color and the types of anthocyanin and copigmentation (Osawa, 1982). The anthocyanins pelargonidin, cyanidin, and delphinidin produce scarlet, crimson, and blue-mauve shades, respectively [absorbance maxima (λ max) of 520, 535, and 546 nm in 0.01% HCl in methanol]. At the pH of cell vacuoles, anthocyanins form chemical bonds either between themselves (intramolecular copigmentation and self association) or with other phenolic molecules (intermolecular copigmentation). The bonds stabilize the pigment and result in an increase in absorbance and a shift in λ max to longer wavelengths (bathochromic shift). Thus, copigmentation results in the "bluing" of red shades. The mechanism of copigmentation is detailed by Brouillard (1983), Brouillard et al. (1989), and Mazza and Brouillard (1990).

The contribution of copigmentation phenomena to fruit color is not well researched, although fruit have an abundance of flavonols and proanthocyanidins suitable for copigmentation (Macheix et al., 1990). In grapes (*Vitis vinifera* L.), flavonol levels are too low to act as copigments, although self-association may occur at very high anthocyanin levels (Moskowitz and Hrazdina, 1981). A case for copigmentation contributing to a difference in skin color has been postulated for *Ilex crenata* Thunb. and *I. rotunda* C.P. Thunb. ex A. Murray in Linnaeus berries, which contain flavonols, and those of *I. sinensis* (Loes.) S.Y. Hu, which do not. *I. sinensis* berry cells have a λ max 12 nm lower than those of *I. crenata* and *I. rotunda* (Ishikura, 1975).

Apple skin contains mainly cyanidin-3-galactoside (Sun and Francis, 1967) and high concentrations of flavonols (quercetin glycosides) and proanthocyanidins, such as catechin (MacRae et al., 1990; Oleszek et al., 1989; Prabha and Patwardhan, 1985). Skin colors ranging from pink-red to deep purple-red are also found in diverse genotypes. It seemed feasible that copigmentation was a factor in producing the different shades of red in apple skin. In the work reported herein, we determined the λ max of cells from the skin of different colored apple genotypes and the molar ratio of flavonols and proanthocyanidins to anthocyanins. We also determined the proportion of red cells in the skin of each genotype. Our results suggest that copigmentation is not a factor in producing differing shades of red. The anthocyanin concentration in the skin and the blending of color from chlorophyll and carotenoid pigments are shown to be important in determining the final red color.

Materials and Methods

Plant material

Five apples genotypes were used, representing a wide color variation: 'Granny Smith' (green background with bronze-red in

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areas exposed to high light intensities), 'Oregon Red Delicious' (deep purple-red), 'Regal Gala' (orange-red), and the breeding lines 4926 and 3827 (both crimson red). The color in each of the genotypes was uniform, not striped. Apples were picked at maturity from research orchards at Hastings, Hawkes Bay, New Zealand, and stored at 4C. Five apples from each genotype were used and analyzed individually.

Color measurements

Zones on each fruit (3 × 3 cm) representing a range of color shade and intensity were labeled with a felt pen so that color measurements were made on the same area of skin used for microscopy. Color measurements were made using the 8-mm-diameter head of a portable tristimulus color analyzer (Chromameter II; Minolta, Ramsey, N.J.) and expressed in Commission Internationale d'Eclairage L*, a*, b* color-space coordinates. The meter was calibrated using the manufacturer's standard white tile.

Skin sections

Skin sections from the labeled areas were cut by hand radially and tangentially using a single-edge blade, mounted in water, and viewed immediately. Only the part of a section that was one cell thick (of cuticle and epidermis) was used for the spectrophotometry of anthocyanin-containing cells.

Microspectrophotometer

Absorbance spectra of individual epidermal cells from the labeled areas were measured with a single-beamed microspectrophotometer (Nanospec 10S; Nanometrics, Sunnyvale, Calif.). The 20-μm-diameter measuring aperture was centered over the vacuole of the cell to be measured. Light intensity measurements were ratioed against those obtained from a colorless reference cell. Cells of each genotype were scanned from 450 to 650 nm to determine λ max. Using the same sections, 50 cells from each genotype were scanned at λ max 550 nm to determine the mean and range of absorbances. Clear vacuoles were used as a reference as above. Photomicrographs of sections of apple skin were made.

Image analysis

Image analysis of 35-mm transparencies at ×20 and ×40 magnification was carried out using version three of the Chromatic Color Image Analysis System (Leading Edge, Bedford Park, South Australia), a video camera (model TK870E RGB; JVC, Japan), and a 33 MHz 80486-based personal computer. The video camera had a 1/2" charge-coupled device, and a 25-mm lens and 5-mm extension tube were used to obtain a video image in which the transparency image just filled the video frame.

Images obtained were subjected to averaging filtration using a 3 × 3 kernel to remove signal noise, and pixel discrimination was carried out for color values that corresponded to the color of vacuoles containing pigment. In some genotypes (e.g., 'Granny Smith') it was necessary to carry out several discrimination procedures. In this case, the overlays produced by each discrimination were mixed until an overlay congruent with all pigmented vacuoles was produced. This overlay was used to calculate the proportion of the frame area pigmented. A similar procedure was used to determine the proportion of the frame occupied by all vacuoles, and the proportion of pigmented to total vacuoles was calculated.

Analysis of pigment composition

Apple skin of desired color was cut from the fruit and the underlying cortical cells were scraped off. Skin was weighed and extracted for analysis of anthocyanins, flavonols, procyanidins, chlorophylls, and carotenoids.

Chlorophyll extraction and estimation. The methods used for determining chlorophyll and total carotenoid content were essentially the same as those used by Knee (1972). Apple peel (0.5 to 1.0 g) was ground to a fine powder using liquid N and extracted with 15 ml of cold acetone. The residue was reextracted with 5-ml aliquots of 80% acetone until clear. The combined extracts were adjusted to 30 ml with 80% acetone and centrifuged at 5000× *g* for 10 min. Absorbance was measured at 645, 652, and 663 nm and a reading was also taken at 700 nm to correct for any turbidity. Chlorophyll content was calculated from the data using the equations of Maclachlan and Zalik (Holden, 1965).

Total carotenoid estimation. A 10-ml aliquot of the acetone extract from the above procedure was taken and 10 ml of petroleum ether (40 to 60C) and 3 ml 50% saturated aqueous ammonium sulfate were added. The upper phase was washed twice with 3 ml of ammonium sulfate, 1 ml of 25% (w/v) potassium hydroxide in methanol was added, and the mixture was stirred for 15 min. The upper phase was washed with 5 ml water until clear. The petroleum ether solution was then dried over sodium sulfate for 1 h before the absorbance was read at 446 nm. A reading was also taken at 550 nm to correct for any turbidity (Goodwin, 1955). Total carotenoid content was calculated, assuming extinction of a 1% solution in a 1-cm light path optical cell ($E^{1\%}$ cm) is 2500.

Flavonol and anthocyanin extraction. Apple peel (0.5 to 1.0 g) was ground to a fine powder using liquid N and extracted with 10 ml 15.0% (v/v) acetic acid in methanol. The residue was reextracted at least twice to remove all of the pigments. The combined extracts were centrifuged at 5000× *g* for 10 min. The extract was rotary-evaporated to almost dryness at 40C and taken up in 0.5 to 1.0 ml of 15% (v/v) acetic acid in methanol. The extract was centrifuged at 10,000× *g* for 5 min before being injected directly into the high-performance liquid chromatograph (HPLC). A solvent delivery control system with an automatic sample injector and a variable-wavelength ultraviolet detector (models 600, WISP 712, and 490; Waters, Milford, Mass.) were used to identify and quantify the flavonoids. The column was 220 × 4.6 mm fitted with a 18 × 3.5 mm guard column (Aquapore RP-18; Applied Biosystems, Foster City, Calif.). Chromatographic traces were recorded using the Waters–Dynamic Solutions Maxima program. Samples (1 to 5 μl) were injected onto a 25C column. A flow rate of 1.0 ml·min^{-1} and a linear 15-min solvent gradient from 5% to 20% acetonitrile in 10% (by volume) acetic acid in water with a 15 min hold at the final concentration was used. Eluted components were monitored at 350 nm for flavonols and 530 nm for anthocyanins. The individual compounds were identified and quantified from weighed amounts of known standards.

Proanthocyanin quantification. The extraction method was the same as for the flavonols and anthocyanins but the solvent used was 100% methanol. The same HPLC system was used as for the flavonols and anthocyanins with the following differences. Solvents used were A) 10.0% (v/v) acetic acid in water and B) water. Samples of 10 μl were injected onto the column, which was maintained at 70C. The flow rate was 1.0 ml·min^{-1} and a linear 47-min solvent gradient of 10% to 82% A, followed by a linear 8-min solvent gradient of 82% to 100% A, and a final hold at 100% for 5 min was used. Eluted components were monitored at 280 nm for proanthocyanidins and 313 nm for phenolic acids.

Results

In vivo light absorbance measurements. Apple skin cells with absorbances of 0.09 to 1.07 (pale to dark red) were scanned, where

possible, for each genotype. For the blushed 'Granny Smith' fruit, only cells from pale to medium red were available (A = 0.22 to 0.45). The mean λ max for 19 cells from five genotypes was 550 + 5.9 nm (Fig. 1). For each of the genotypes, there was a broad λ max, particularly for 'Oregon Red Delicious' and 'Regal Gala', which had the darkest skin color. In the blushed 'Granny Smith' fruit, the λ max range was 550 to 556 nm, with an average λ max of 553 ± 0.0 nm. There was no trend of increasing λ max with darker red skin. 'Oregon Red Delicious' had a 2-nm lower average λ max than 'Granny Smith'. A correlation of absorbance to mean λ max was carried out for apple cells, and no relationship was found ($r^2 = 0.10$).

Copigmentation. Quercetin glycosides and proanthocyanidins function strongly in copigmentation and, thus, bathochromic shifts in λ max (Osawa, 1982). High concentrations of both of these classes of compounds were present in each of the genotypes (Table 1). Flavonols were generally 1×10^{-2} M for each of the genotypes; proanthocyanidins ranged from 0.64×10^{-2} M for 3827 to 2.2×10^{-2} M for 'Oregon Red Delicious'. Anthocyanins showed a wider range (7-fold), from $\approx 0.2 \times 10^{-2}$ M for 4926 and 3827 to 1.5×10^{-2} M for 'Oregon Red Delicious'. The blushed 'Granny Smith' fruit contained minimal amounts of anthocyanin. These values were used to calculate the ratio of flavonols and proanthocyanidins to anthocyanins. Higher ratios indicate a greater potential for copigmentation. The genotypes 4926 and 3827 had a ratio of ≈10, whereas the darker red 'Regal Gala' and 'Oregon Red Delicious' had ratios of ≈3 and 2, respectively. 'Granny Smith' by far had the highest ratio of 208.

Anthocyanin distribution in skin. Thin tangential sections of apple skin showed a nonuniform distribution of anthocyanin among epidermal cells for all five genotypes when viewed at magnification ×400 (Fig. 2). Very dark, red cells were adjacent to naturally pale cells. However, within a cell, the vacuole(s) appeared uniform in color.

Transmission at 550 nm was measured for 50 cells for each genotype and absorbance was calculated. A colorless cell in each section was used as a reference. A histogram of vacuole absorbances was plotted for each genotype (Fig. 3). All genotypes, in the region of apple skin examined, showed some cells without anthocyanins in their vacuoles. This pattern was greatest for 'Granny Smith' (25% without anthocyanins). In the blushed area of 'Granny Smith' skin, the λ max of cells was 0.45 (2% of cells) and, in 84% of the cells, it was <0.20. 'Oregon Red Delicious' and 'Regal Gala' showed a wide range of absorbance, from 0.0 to 0.85 and 1.20 respectively. For 'Regal Gala', 78% of the cells was >0.65 absorbance and 44% for 'Oregon Red Delicious'. The genotypes 3827 and 4926 had an absorbance distribution between those of 'Granny Smith' and 'Regal Gala' and 'Oregon Red Delicious', with 20% and 4% of the cells, respectively, with absorbance >0.65. Correspondingly, skin color of genotypes 3827 and 4926 was lighter red than that of 'Regal Gala' and 'Oregon Red Delicious' but darker than that of 'Granny Smith'. Thus, increasing redness of skin could be accounted for by a higher proportion of dark red cells.

'Oregon Red Delicious' is much darker than 'Regal Gala', although 'Regal Gala' has the higher proportion of higher absorbance cells. A possible explanation for this discrepancy is that 'Oregon Red Delicious' has its anthocyanin in up to three layers of cells in the epidermis (Fig. 2), whereas, in 'Regal Gala', anthocyanin is limited to one layer, and occasionally two. Alternatively, the darker color could be accounted for by the higher absorbance at shorter wavelengths (Fig. 1) of pigments, either in the vacuole or in the cytoplasm behind the vacuole.

A striking feature of the epidermal sections is the differences in vacuolar size within the cells. Cell size is similar for all genotypes,

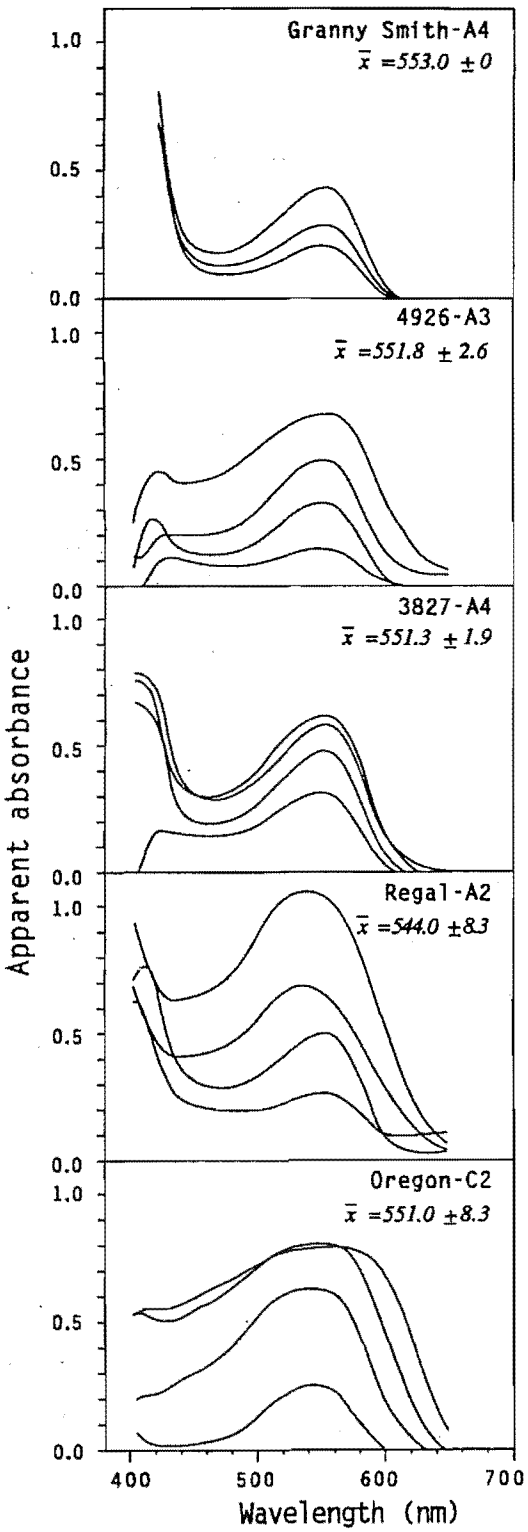

Fig. 1. Reflectance spectra (using a microspectrophotometer) of anthocyanin-containing vacuoles for five apple genotypes of specified skin areas.

Table 1. Concentrations of anthocyanins, flavonols, and proanthocyanidins and the ratio of flavonols and proanthocyanidins and anthocyanins in relation to copigmentation potential of five apple genotypes.

Genotype	Anthocyanin (M)	Flavonol (M)	Proanthocyanidin (M)	Ratio (F + P)/A
Granny Smith	0.09×10^{-3}	1.1×10^{-2}	0.78×10^{-2}	208
4926	0.27×10^{-2}	1.7×10^{-2}	0.86×10^{-2}	9.5
3827	0.18×10^{-2}	1.3×10^{-2}	0.64×10^{-2}	10.7
Regal Gala	0.66×10^{-2}	1.2×10^{-2}	1.1×10^{-2}	3.5
Oregon Red Delicious	1.50×10^{-2}	1.7×10^{-2}	2.2×10^{-2}	2.6

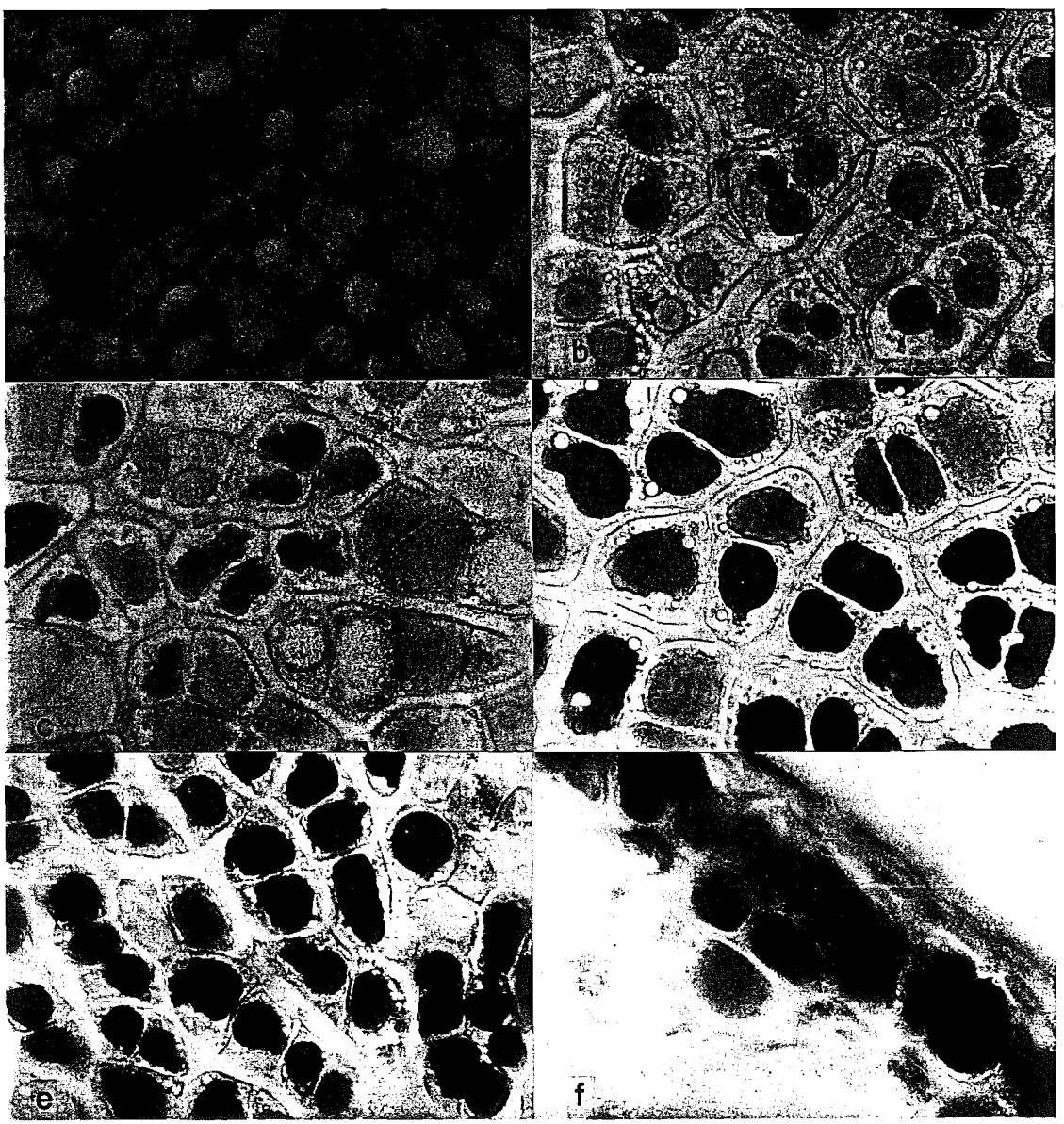

Fig. 2. Anthocyanin distribution in tangential (a–e) and transverse (f) sections of apple skin of (a) 'Granny Smith', (b) 3827, (c) 4926, (d) 'Regal Gala', and (e and f) 'Oregon Red Delicious'. Magnification ×236.

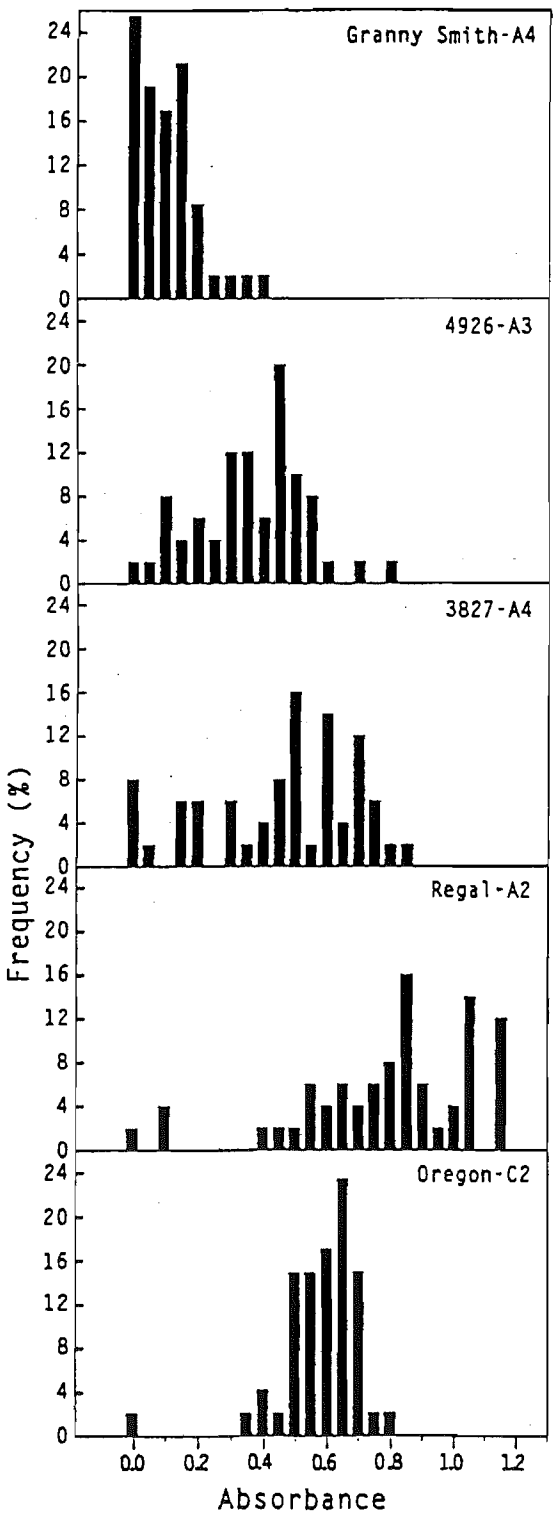

Fig. 3. Frequency (%) of cells of varying redness (550 nm) in tangential section of skin of specified areas of five apple genotypes. For 'Granny Smith', 50 cells were measured. For each of the other genotypes, 100 cells were measured.

but there are up to 4-fold differences in the area covered by vacuoles (Table 2). Vacuoles account for ≈50% of the surface area in 'Regal Gala', 'Oregon Red Delicious', and 'Granny Smith'; 3827 and 4926 have ≈20% and 30%, respectively, of the cell area as vacuoles. Thus, 3827 and 4926 do not have the same potential for reddening as do the other three genotypes.

Influence of chlorophyll and carotenoids on red skin color. The microspectrophotometric studies on absorbance of individual cells have shown no major differences in λ max; however, chromameter measurements on the skin of apple genotypes have shown large differences in L*, a*, b* values (Table 3), and differences in shade of red are apparent to the eye. Surprisingly, the darkest skinned genotype, 'Oregon Red Delicious', has a lower a* value than genotypes 3827 and 4926. The zones of apple skin used for microspectrophotometry were those typical of the mature fruit, and, in the case of 'Granny Smith', a blushed zone was used. There was no significant correlation between anthocyanin content and a* values, ($r^2 = 0.09$), a result that is unexpected since a* is a measure of redness. Neither b* or $\tan^1$ b*/a* correlated with anthocyanin content. Lightness values (L*) ranged from 47.6 for 'Granny Smith' to 33.9 for 'Oregon Red Delicious'. A coefficient of determination ($r^2 = 0.68$) was obtained for the regression of anthocyanin content on L* values. The five genotypes contain widely different levels of chlorophyll, carotenoids, and anthocyanins (Table 3). 'Granny Smith' is high in chlorophyll, whereas 'Regal Gala' contained no chlorophyll. Genotypes 3827, 4926, and 'Oregon Red Delicious' have levels of chlorophyll ≈25% that of 'Granny Smith'. Carotenoid levels were highest in 'Granny Smith', 'Regal Gala', and 4926 and lowest in 'Oregon Red Delicious'.

Discussion

Microspectrophotometer studies, although few in number, have shown bathochromic shifts in λ max attributable to copigmentation. Petals of orange and red *Azalea* showed a 10-nm bathochromic shift attributable to the presence of flavonols in red *Azalea* (Asen et al., 1971b). In *Ilex* berries, flavonols in *I. crenata* gave rise to putative copigmentation and an increased λ max of 13 nm compared with the flavonol-free *I. chinensis* (Ishikura, 1975).

For the apple skin cells measured in this work, mean λ max did not differ significantly among genotypes. There was no trend of increasing λ max with darker red skin. Although the molarity of flavonols and proanthocyanidins in apple is in the range that one would consider to be effective in copigmentation in floral tissue (Asen et al., 1972; Chen and Hrazadina, 1981) and in vitro studies, there was little difference in copigmentation ratios between genotypes, and copigmentation ratios in the more deeply colored genotypes were lower than in the lightly colored genotypes. These results, together with the similarity of λ max for the five genotypes, show that copigmentation is not an explanation for the different shades of red. Although a mean λ max of 550 nm was observed for the apple cells, this was considerably higher than the 523-nm λ max for cyanidin-3-galactoside in aqueous solution at pH 3.0. This result suggests that copigmentation is a factor accounting for the higher λ max in all of the genotypes, but it is not an explanation for genotype differences in shade of red.

The spectral absorbance of anthocyanins is also influenced by pH. Between pH 3.5 and 5.5, there is an equilibrium between the red flavylium cation and the blue-purple quinonoidal base. Increasing pH results in a shift to the quinonoidal base, a higher λ max, and loss in absorbance (Macheix et al., 1990). The pH of apple epidermal tissue was determined as 3.6 to 4.0 (unpublished

data), but there are no published measurements on pH of skin of different genotypes. The relative uniformity of λ max for the different genotypes argues against pH as an explanation for the different colors of red in different genotypes.

The darker colored 'Oregon Red Delicious' and 'Regal Gala' had a higher proportion of cells with higher absorbances at 550 nm, larger vacuoles, and, in the case of the former genotype, up to three layers of red cells compared to only one in genotypes 4926 and 3826.

The apparently random distribution of colored cells in apple skin is a striking feature of this work. A similar distribution of red color in apple skin had been previously reported but not quantified by Dayton (1959), Dickinson and White (1986), and Misic and Tesovic (1971). Variation in the color of individual adjacent cells has also been observed in rose petals (Asen et al., 1971a), where the color difference was attributable to different vacuolar pHs of adjacent cells. The lack of discernible pattern in anthocyanin distribution in apple skin cells makes it difficult to suggest a cytological or developmental mechanism. Furthermore, for apple cells, light is one of the main determinants of anthocyanin production, yet adjacent cells would have the same light environment. The mechanism for producing the observed differences in cellular anthocyanin concentration remains unknown.

Although there were higher proportions of red cells in the skin of more darkly colored genotypes, such as 'Oregon Red Delicious', no correlation was observed between anthocyanin content and a* values for the five genotypes. Singha et al. (1991) reported coefficients of determination for selected regression models relating chromaticity values to anthocyanin content. They found a poor correlation between a* and anthocyanin levels ($R^2 = 0.10$). Correlations between anthocyanin levels and chromaticity values $(a^*/b^*)^2$ and $L^*(a^*/b^*)^2$ ($R^2 = 0.81$) were achieved with a separate equation for each strain of 'Delicious'. The relationships, however, are empirically derived and have no theoretical basis.

In some fruit {e.g., tomato [*Lycopersicon esculentum* var. *cerasiforme* (Duval) A. Gray] and cranberry (*Vaccinium macrocarpon* Ait.)}, there is good agreement among visual scores, red pigment content, and Hunter values (Francis and Clydesdale, 1970; Larrigaudiere et al., 1991). For tomato, the a* value is sufficient to characterize maturity stages. However in each of these fruit, the pigment composition and pigment changes are simpler, whereas in apple skin, there are three pigment classes (chlorophyll, carotenoids, and anthocyanins).

For ripening peaches [*Prunus persica* (L.) Batsch.], major changes in color were reflected by an increase in a* but no change in b* (Byrne et al., 1991; Delwiche and Baumgardner 1985). However, the increased a* value was brought about by two pigment changes—a decrease in chlorophyll and an increase in anthocyanin. Similarly, for the surface of watermelons [*Citrullus lanatus* (Thunb. Matsum. & Nakai)], an increase in a* values during ripening is brought about by a loss of chlorophyll. The carotenoid levels and, thus, the b* value is constant, but the fruit appear more orange because the chlorophyll loss unmasks the carotenoids (Corey and Schlimme, 1988). In papaya (*Carica papaya* L.), in which ripening is characterized by the fruit turning yellow and an increase in b* values, small green flecks considerably reduced the a* values (Peleg and Gomez Brito, 1975). Genotypes such as 'Oregon Red Delicious', which have high anthocyanin levels, and, therefore would be expected to have a higher a* value, also have high chlorophyll levels, which reduce the a* value.

The modifying effect of chlorophyll on anthocyanins has also been monitored for eggplant (*Solanum melongena* L.), in which the darkest purple fruit had high levels of chlorophyll and anthocyanins (Nothmann et al., 1976). Likewise, in petals from tulip (*Tulipa gesnerana* L.) and chrysanthemum [*Dendranthema grandiflorum* (Ramat.) Kitamura], carotenoids in the presence of anthocyanins modify the appearance to red-orange or bronze (Nieuwhof et al., 1989; Teynor et al., 1989). As Knee (1980) observed, evidence is lacking on the extent to which differences in pigment composition are apparent as color differences to the eye.

In conclusion, an increase in skin darkness in apples could be accounted for by increased anthocyanin concentration from a greater proportion of darker red vacuoles, larger vacuoles, and several layers of red cells. A change in the hue of the red coloration of apple skin from orange-red to bronze or purple-red is more likely to result from the visual blending of chlorophyll, carotenoids, and anthocyanins than from differences in copigmentation.

Table 2. Percentage of total skin area covered by vacuoles and percentage of anthocyanin-containing vacuoles in five apple genotypes differing in red skin color. Two photographic slides were measured for each genotype.

Genotype	Total vacuole area (%)	Anthocyanin-containing vacuoles (%)
3827	20, 13	100, 92
4926	36, 27	97, 96
Granny Smith	54, 47	15, 17
Regal Gala	41, 49	97, 100
Oregon Red Delicious	47, 51	98, 98

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Table 3. Chromaticity values (L*, a*, b*) and pigment content of apple skin of five genotypes differing in visual appearance.

Genotype	L*	a*	b*	Chlorophyll ($\mu g \cdot g^{-1}$ fr wt)	Carotenoids ($\mu g \cdot g^{-1}$ fr wt)	Cyanidin-3-galactoside ($mg \cdot g^{-1}$ fr wt)
Granny Smith	47.6	3.5	16.2	170.8	21.7	0.025
3827	39.5	20.2	6.8	37.7	10.4	0.5
4926	45.4	30.8	14.8	61.3	15.5	0.8
Regal Gala	35.8	9.2	1.6	0	16.3	1.9
Oregon Red Delicious	33.9	7.1	0.8	40.7	8.6	4.1

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Developmental Changes in the Concentration and Composition of Flavonoids in Skin of a Red and a Green Apple Cultivar

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Abstract: Flavonoids from the skin of Granny Smith, a green apple cultivar, and Splendour, a red apple cultivar, were quantified by high-performance liquid chromatography for two seasons (1989–1990 and 1990–1991). Both cultivars contained a similar composition and concentration of quercetin glycosides and proanthocyanidins. Splendour also synthesised cyanidin glycosides during ripening. Quercetin glycosides and proanthocyanidins were highest in the skin of very young fruit of Granny Smith and decreased by 50% during fruit development. In Splendour, concentrations of quercetin glycosides and proanthocyanidins in the skin decreased by 50% from early to mid-season but then increased during ripening. Cyanidin glycosides in Splendour increased to about 1 mg g^{-1} fresh weight during ripening. There were significant differences between the two cultivars but not between years. Total amount of flavonoids increased throughout the season as fruit surface area increased. For Granny Smith there was an estimated net synthesis per apple of 0·16 mg day^{-1} quercetin glycosides, 0·1 mg day^{-1} proanthocyanidins and for Splendour a net synthesis per apple of 0·28 mg day^{-1} quercetin glycosides, 0·21 mg day^{-1} proanthocyanidins and during ripening 0·21 mg day^{-1} cyanidin glycosides. Relative proportions of major quercetin glycosides and proanthocyanidins were stable during fruit development. For Splendour, however, cyanidin glycoside synthesis was accompanied by a corresponding increase in quercetin glycoside and proanthocyanidin synthesis. The data suggest a coordinate regulation of enzymes in the flavonoid biosynthetic pathway during fruit development.

Key words: apple skin, fruit development, anthocyanins, cyanidin glycosides, quercetin glycosides, proanthocyanidins.

INTRODUCTION

Flavonoids are an important component of apple skin, being present at 10–30 g kg^{-1} DM. The main flavonoids are quercetin glycosides, cyanidin glycosides, epicatechin and its polymers (Lancaster 1992). A diversity of biological functions has been proposed for these compounds. Cyanidin glycosides are responsible for the red colouring in apples and function as attractants in fruit dispersal. Flavonoid molecules may also protect cells by absorbing potentially damaging ultraviolet (UV) radiation. A role as defence chemicals against

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pathogens and predator has also been suggested for flavonoids (Luckner 1990), hence their high concentration in the skin of fruit.

Cyanidin glycosides, quercetin glycosides and proanthocyanidins in apple skin also have economic and aesthetic value. Desirable colouration is produced by cyanidin glycosides stabilised or co-pigmented by quercetin glycosides and proanthocyanidins (Lancaster *et al* 1994) whilst an undesirable brown colouration in processed apples is produced by the action of polyphenol oxidase on flavanols and proanthocyanidins (Burda *et al* 1990). Proanthocyanidins and their polymers interact with the glycoproteins of the salivary and mouth mucosa and cause the sensation of astringency characteristic of fermented apple beverages (Lea and Timberlake 1974).

The authors are interested in the regulation of the biosynthetic pathway to quercetin glycosides, proanthocyanidins and cyanidin glycosides in apple skin. The role of light in the regulation of anthocyanin biosynthesis in apple skin has been studied, but there has been little work on developmental regulation and most of this has focused on regulation during fruit ripening (Lancaster 1992). In Cox's Orange Pippin anthocyanin levels per apple increased three-fold during the final month of ripening (Knee 1972). Quercetin glycoside concentration decreased during the maturation of Golden Delicious apples (Workman 1963; Gorski and Creasey 1977), and subsequently increased during storage (Gorski and Creasey 1977). Burda *et al* (1990) followed enzymic browning during the development of Rhode Island Greening apples and showed that the major flavonoid, epicatechin, was high in the skin of young fruit, decreased and then remained relatively constant during maturation and storage. Average levels of epicatechin, procyanidin B2, quercetin glycosides, phloretin glycosides and chlorogenic acid were recorded for stored apple skin.

There is no published work on the concentration of all major flavonoids during fruit development despite their close biosynthetic relationship and most of the existing work has focused on cultivars with green- or yellow-, but not red-skinned fruit. This paper reports on developmental changes in the concentration and total amount of cyanidin-3-galactoside, quercetin glycosides and proanthocyanidins in the skin of fruit from red and green apple cultivars.

EXPERIMENTAL

Plant material

Fruits of the cultivars Granny Smith and Splendour were obtained from the Lincoln University Research Orchard (Christchurch, New Zealand). Samples of five apples per cultivar were collected on 12 December, 5, 26 January, 15 February, 16, 29 March and 12 April in 1989–1990 and 11, 27 December, 14 January, 14 February, 4, 21 March and 10 April in 1990–1991. At each sample date the fruit weight and diameter was recorded. Fruit diameter increased from about 38 to 85 mm between the start and finish of sampling. Peel was removed carefully, taking care to scrape away underlying cortex, and weighed. Half the peel from each fruit was used for flavonoid determination.

Extraction of flavonols and anthocyanins

Apple peel (0·5–1·0 g) was ground to a fine powder in liquid nitrogen and then extracted with 10 ml 150 ml litre^{-1} acetic acid in methanol. The residue was re-extracted at least twice, to remove all the colour and the combined extracts centrifuged at 5000 × *g* for 10 min. The extract was concentrated to almost dryness at 40°C and taken up in 0·5–1·0 ml 150 ml litre^{-1} acetic acid in methanol and centrifuged at 10000 × *g* for 5 min before injecting directly onto the high-performance liquid chromatograph (HPLC).

Quantification of flavonols and anthocyanins

A Waters 600 solvent delivery control system with a Waters WISP 712 automatic sample injector and a Waters 490 variable wavelength UV detector were used to identify and quantify the flavonoids. The column was a 220 × 4·6 mm Applied Biosystems Aquapore RP-18 fitted with an 15 × 3·2 mm Applied Biosystems Aquapore RP-18 Guard column. Chromatographic traces were recorded using the Waters/Dynamic Solutions 'Maxima' program. Solvents used for elution were (A) 100 ml litre^{-1} acetic acid in water and (B) acetonitrile. Deaeration was achieved by vacuum filtration through a 0·22 μm filter, rapid sparging with helium (100 ml min^{-1} for 10 min) and constant slow bubbling of helium into capped, vented solvent reservoirs (5 ml min^{-1}). Samples (5 μl) were injected on to the column which was maintained at 30°C using a Waters column heater. A flow rate of 1·0 ml min^{-1} and a linear 20 min solvent gradient from 0 to 20% acetonitrile, with a 10 min hold at the final concentration was used. The column was returned to initial solvent composition over 1 min and re-equilibrated for 10 min before the next analysis. Eluted components were monitored at 350 nm for flavonols and 530 nm for anthocyanins. The individual compounds were identified and quantified by comparison with standard solutions of known concentration.

Quantification of proanthocyanidins

The method of extraction was the same as that for the flavonols/anthocyanins but the solvent used throughout

was 100% methanol. The same HPLC system was used as for the flavonols/anthocyanins. Eluted components were monitored at 280 nm for proanthocyanidins and 313 nm for phenolic acids.

Statistical analysis

Concentration and total amount of quercetin glycosides, proanthocyanidins and cyanidin glycosides were measured for each fruit. Fruit diameter was used to estimate surface area, assuming spherical shape, and total amounts of each compound calculated for individual fruit. Standard ANOVA established significant differences among varieties and over time, so subsequently each variety was analysed separately. Concentrations for some minor compounds, and amounts per apple for all compounds, were log transformed before analysis to stabilise variances over the season. The angular transformation was applied to the relative amounts (porportions) of the individual components of quercetin glycosides and proanthocyanins. Graphs were drawn using the scale used for analyses so that least significant differences (LSD) at the 5% significance level could be used to indicate experimental precision. LSD used are those appropriate for comparing means at two dates for a given component. For Fig 1(b), where concentrations of cyanidin glycosides were near zero before March each year this data was omitted for computation of LSD.

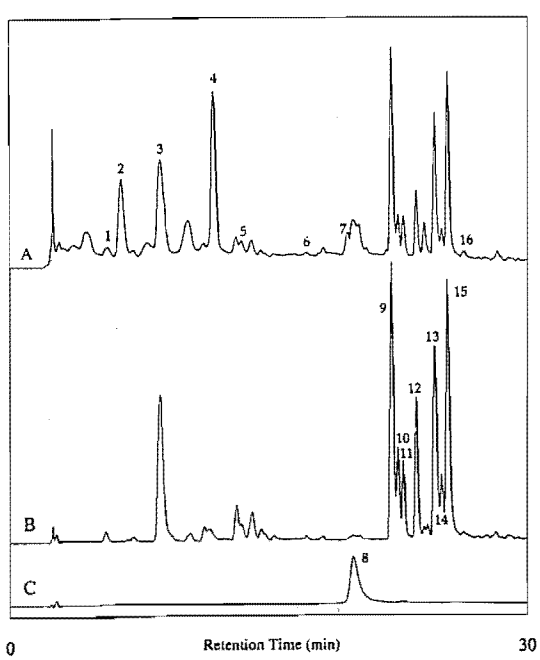

Fig 1. Typical chromatogram of flavonoid pigments extracted from a sample of skin of the apple variety Splendour. The three wavelength profiles shown are appropriate to optimal detection of (A) proanthocyanins (λ_{max}, 280 nm), (B) quercetin glycosides (λ_{max}, 350 nm) and (C) anthocyanins (λ_{max}, 530 nm). Peak assignments refer to the elution order shown in Table 1.

RESULTS

Identification of flavonoids

Cyanidin glycosides, quercetin glycosides and proanthocyanidins were resolved by reverse-phase HPLC (RP-HPLC) (Fig 1). Initially quercetin glycosides were identified by co-chromatography with authentic standards, then individual glycosides were collected from the HPLC column and the glycoside identified by hydrolysis and co-chromatography on thin-layer chromatography (TLC) with authentic sugar standards (Webby R unpublished). The anthocyanin, proanthocyanidins, phloridzin and chlorogenic acid were identified by their co-elution with known standards (Table 1). Gallocatechin has been reported in apples and identified by TLC and spot test (Mosel and Herrmann 1974). Attempts to purify and identify conclusively this compound by nuclear magnetic resonance have been unsuccessful to date and in this paper identification is by comparison to the retention time of an authentic gallocatechin standard.

Levels of flavonoids in developing fruit

Quercetin glycosides were the major flavonoids in Granny Smith and Splendour apples at a concentration of 4–7 mg g^{-1} and 2·5–5·5 mg g^{-1} (13–23 and 8–18 g kg^{-1} DM), respectively (Fig 2). Proanthocyanidins were

TABLE 1

Retention times of the anthocyanins, quercetin glycosides and proanthocyanidins of apple fruit on RP-HPLC

Elution order	*Flavonoid*	*Retention time (min)*	λ_{max} *(nm)*
1	Catechin[a]	5·9	280
2	Procyandin B2[a]	6·6	280
3	Chlorogenic acid[a]	8·8	280
4	Epicatechin[a]	11·9	280
5	Gallocatechin[a]	13·9	280
6	Dihydroquercetin[a]	17·6	280
7	Proanthocyandin B5[a]	19·6	280
8	Cyanidin-3-galactoside[a]	19·9	530
9	Quercetin-3-galactoside[a]	22·2	350
10	Quercetin-3-rhamnoglucoside[b]	22·5	350
11	Quercetin-3-glucoside[a]	22·8	350
12	Quercetin-3-xyloside[a]	23·6	350
13	Quercetin-3-arabinopyranoside[b]	24·3	350
14	Quercetin-3-arabinofuranoside[b]	24·6	350
15	Quercetin-3-rhamnoside[a]	25·4	350
16	Phloridzin[a]	27·1	280

[a] Co-chromatographed with authentic standards.
[b] Identified by sugar and co-chromatography with authentic sugar standards.

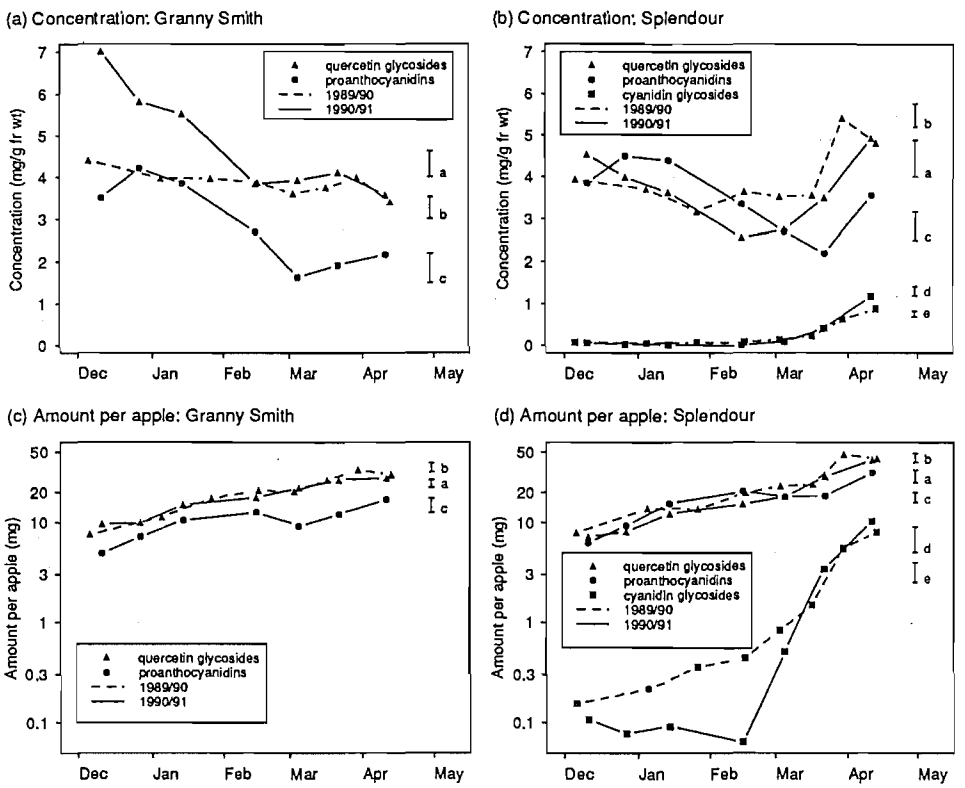

Fig 2. Changes in (a,b) the concentration and (c,d) total amount of ▲, quercetin glycosides; ●, proanthocyanidins; and ■, cyanidin glycosides of (a,c) Granny Smith and (b,d) Splendour apples during the season of ---, 1989–1990; and ———, 1990–1991.

present at a concentration of 1·5–4·5 mg g^{-1} for Granny Smith and 2–4·5 mg g^{-1} for Splendour. Cyanidin glycosides were present in Splendour at a concentration of 0–1·3 mg g^{-1} FW (Fig 2(a) and (b)).

The concentration of quercetin glycosides and proanthocyanidins changed significantly during fruit development. In Granny Smith apples concentration was higher in young fruit, and decreased significantly from December to April. This was particularly so for 1990–1991, when quercetin glycosides and proanthocyanidins decreased by about 50% between December and April. In Splendour apples, concentrations of quercetin glycosides and proanthocyanidins decreased during the season, but increased from mid-March onwards. In 1989–1990 an end of season decrease in quercetin glycosides occurred, but in 1990–1991 the last two sampling dates were too far apart to detect a small peak in quercetin glycosides if it did occur. An increase in anthocyanin concentration in Splendour apples from 0 to 1 mg g^{-1} occurred between mid-March and mid-April for both years. Analysis of variance showed that the two cultivars were significantly different at the 5% level but that differences between the 2 years, for a given cultivar, were not.

Although the concentration of quercetin glycosides and proanthocyanidins in the skin decreased during the season their total amount per fruit increased steadily as the fruit surface area increased (Figs 2(c) and (d)). Linear regressions of the (log) amounts of quercetin glycosides and proanthocyanidins against time were computed and showed no significant differences between the two years, being similar for both cultivars over the whole season. Rates were parallel but with significantly different intercepts on the y-axis (Figs 2(c) and (d)). Cyanidin glycosides in Splendour showed a significantly different pattern of accumulation, being negligible until early March then rising rapidly.

Final amounts of quercetin glycosides were 30 mg (1989–1990) and 28 mg (1990–1991) for Granny Smith fruit and 43·7 mg (1989–1990) and 43·0 mg (1990–1991) for Splendour fruit. Proanthocyanidins reached a final amount of 17 mg and 31 mg for Granny Smith and Splendour rspectively. Assuming synthesis, but not breakdown, this corresponds to the synthesis of 0·16 mg day^{-1} of quercetin glycoside for Granny Smith and 0·32 mg day^{-1} for Splendour. Proanthocyanidin synthesis was calculated at 0·1 mg day^{-1} for Granny Smith and 0·21 mg day^{-1} for Splendour. Cyanidin glycoside

synthesis was 0·21 mg day^{-1} for Splendour from mid-March onwards.

Relative amounts of quercetin glycosides

The amount of each quercetin glycoside proportion of total quercetin glycosides was calculated for Granny Smith and Splendour for both years (Fig 3). Both cultivars synthesised the same quercetin glycosides, although the relative proportions differed. For Granny Smith the four main glycosides, comprising about 840 mg g^{-1} total glycosides, were, in descending order, quercetin-3-galactoside, -3-arabinofuranoside, -3-rhamnoside, -3-xyloside. The ranking was the same for both years, although in 1989–1990 quercetin-3-galactoside and quercetin-3-rhamnoside exhibited a greater proportion. Quercetin-3-glucoside, quercetin-3-rhamnoglucose, quercetin-3-arabinopyranoside and an unidentified quercetin glycoside accounted for about 160 mg g^{-1} of total glycosides. For Splendour the four main glycosides, comprising about 900 mg g^{-1} of the total glycosides, were similar to Granny Smith except that the quercetin-3-rhamnoside was a higher proportion than the quercetin-3-arabinofuranoside. The ranking was the same for both years. The ranking of the minor glycosides was similar to that for Granny Smith.

There were fluctuations in the proportions of the individual quercetin glycosides during the growth of the apple fruit and, in particular changes, at the end of the season. Quercetin-3-glucoside increased by about 8% at the end of the season for Splendour in both years and Granny Smith in 1990–1991. For both years Splendour showed an end of season increase in quercetin-3-galactoside and a corresponding decrease in the relative proportions of quercetin-3-rhamnoside. Quercetin-3-glucoside increased and quercetin-3-rhamnoglucoside decreased at the end of the season for both cultivars in 1990–1991, but not in 1989–1990.

Relative amounts of proanthocyanidins

The main proanthocyanidins in both Granny Smith and Splendour were B2 and B5 plus their monomeric precursor epicatechin (Fig 4). Catechin and putative gallocatechin were minor components. The proportion of unidentified proanthocyanidins reached 250–300 mg g^{-1} in both cultivars. Proanthocyanidin profiles were similar for both cultivars, although there were significant changes during the season. Proanthocyanidin B2 (epicatechin-4B → 6-epicatechin) was the main compound between December and March; after March proanthocyanidin B5 (epicatechin-4B → 8-epicatechin)

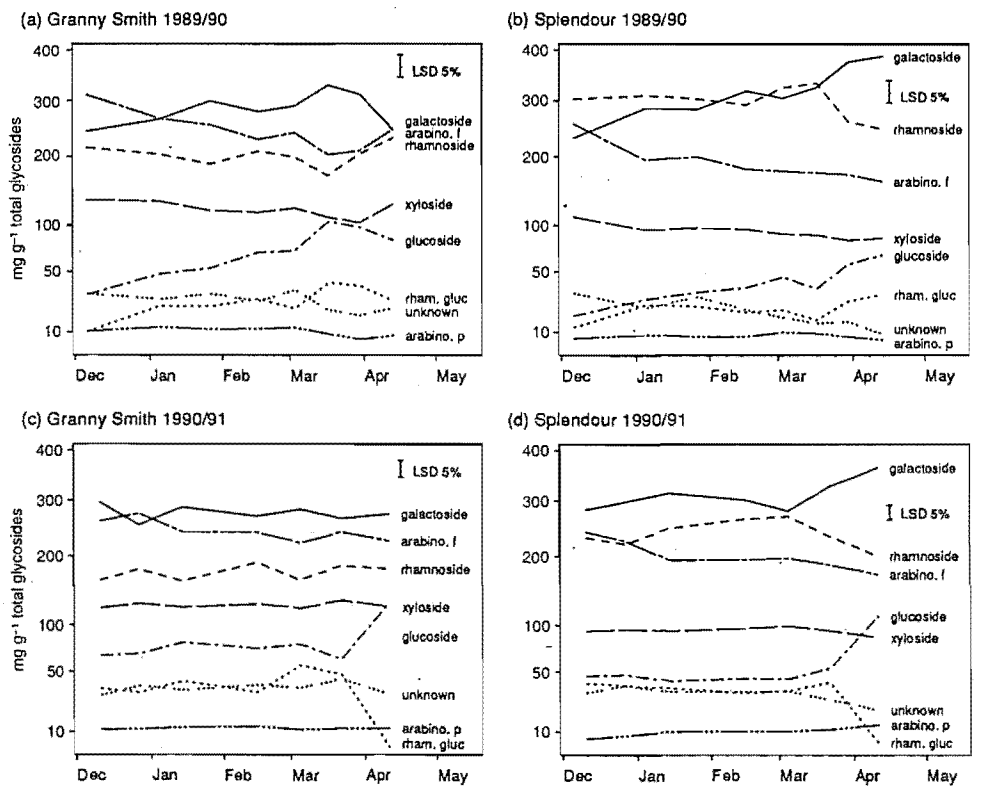

Fig 3. Relative proportion (mg g^{-1} total glycosides) of individual quercetin glycosides for (a,c) Granny Smith, and (b,d) Splendour apples during the season of (a,b) 1989–1990, and (c,d) 1990–1991.

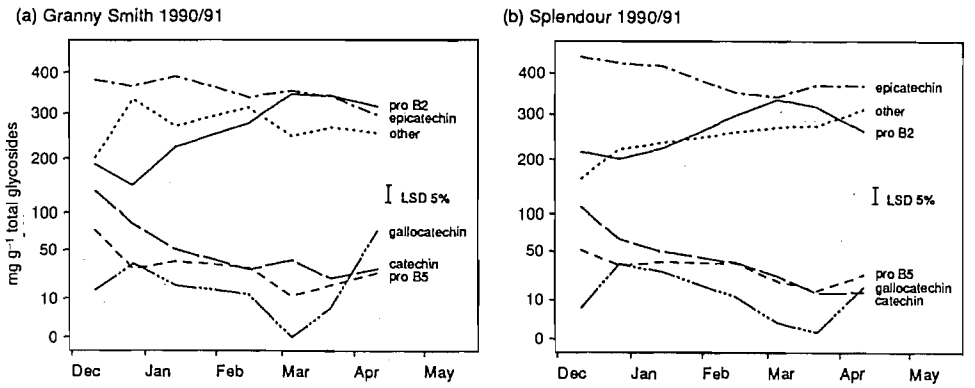

Fig 4. Relative proportions (mg g^{-1} total glycosides) of individual proanthocyanidins for (a) Granny Smith, and (b) Splendour apples during the season of 1990–1991.

predominated. Gallocatechin was proportionately highest in December and in April, being lower during the middle of the season.

DISCUSSION

This paper reports on an improved HPLC procedure which allows separation and quantification of cyanidin galactoside, seven quercetin glycosides, epicatechin, catechin, gallocatechin standard and proanthocyanidins B2 and B5. Previous published methods have been unable to resolve all the quercetin glycosides (Oleszek *et al* 1988; McRae *et al* 1990) or proanthocyanidins (Oleszek *et al* 1988; Putman and Butler 1989; de Silva *et al* 1990).

Using this procedure the two isomers of quercetin (-3-arabinopyranoside and -3-arabinofuranoside) were identified. A further unknown, but minor quercetin glycoside was present at too low a concentration to allow identification. Gallocatechin has been isolated and identified previously by TLC in only two cultivars of apple, Golden Delicious and Schöner von Boskoop (Mosel and Herrmann 1974) and now we report a flavonoid co-chromatographing with authentic gallocatechin in Granny Smith and Splendour. In apple skin the flavonoids which accumulate are quercetin glycosides, epicatechin, procyanidins and in some instances cyanidin glycosides. The galactoside of both quercetin and cyanidin was the predominant glycoside.

The main difference in flavonoid composition between Granny Smith and Splendour apples was the presence of cyanidin-3-galactoside in the latter, red cultivar. Both cultivars contained the same precursor profile of quercetin glycosides and proanthocyanidins, suggesting that in Granny Smith fruit the enzymes converting leucocyanidin to cyanidin glycosides are not expressed. Nonetheless, cyanidin-3-galactoside, and thus reddening, is observed in juvenile fruit. Although both cultivars contained the same quercetin glycosides, there were differences between the relative proportions of individual glycosides. In Granny Smith quercetin galactoside and arabinoside were the principal glycosides, with the 3-rhamnoside a little lower in concentration than the arabinofuranoside whilst in Splendour the 3-arabinofuranoside and rhamnoside were in reversed order. In eight additional cultivars galactoside/glucoside and arabinoside were shown to be the principal quercetin glycosides, with the rhamnoside in a much lower proportion (McRae *et al* 1990). In Golden Delicious, Empire and Rhode Island Greening quercetin galactoside was the principal glycoside, followed by quercetin rhamnoside and quercetin arabinoside (Burda *et al* 1990).

The quercetin glycoside profile for Granny Smith and Splendour was similar for both years, particularly in mid-season. Differences in the relative proportions of quercetin glycosides occurred in early (December) and late season (April), eg the relative proportion of quercetin galactoside in Granny Smith decreased in April 1989–1990 but not in April 1990–1991.

The proanthocyanidin profile was similar for Granny Smith and Splendour, particularly for epicatechin and the minor compounds catechin, gallocatechin and procyanidin B5. In Granny Smith procyanidin B2 increased in relative proportion at the end of the season compared with Splendour, whilst in Golden Delicious, Empire and Rhode Island Greening epicatechin was more abundant than procyanidin B2 at the end of the season (Burda *et al* 1990).

The concentrations of flavonoids detected in Granny Smith and Splendour fruit skin were higher than those previously reported for other cultivars. Total quercetin glycoside levels were between 3 and 7 mg g^{-1}, total proanthocyanidins between 1·5 and 5 mg g^{-1} and cyanidin glycoside at 0–1·1 mg g^{-1}. Previously reported values of quercetin glycosides range from 0·9 mg g^{-1} (Workman 1963) and 1·09 mg g^{-1} (Burda *et al* 1990) to 1·6 mg g^{-1} (McRae *et al* 1990). Proanthocyanidin values ranged from 1·1 to 1·4 mg g^{-1} (Mosel and Herrmann 1974; Burda *et al* 1990; McRae *et al* 1990).

Since all these values are from different cultivars grown at different locations it is difficult to specify causal factors. McRae *et al* (1990) found that growing conditions had a limited effect on polyphenol profiles but did not discuss effects on concentrations *per se*. Flavonoid concentrations are known to increase in response to light intensity and quality and, because New Zealand has high incident light with a high UV component, it is possible that this will lead to higher levels of flavonoids in apple skins.

The concentration of quercetin glycosides and proanthocyanidins was highest in early season and had significantly decreased by mid season for Granny Smith and Splendour. High concentrations of proanthocyanidins in young fruit was also reported by Burda *et al* (1990) and Mosel and Herrmann (1974). In Splendour there was an increase in concentration of all flavonoids during maturation. Gallocatechin was a very minor component of total proanthocyanidins during mid-season but at the end of the season it increased to about 8% in Granny Smith and 3% in Splendour. Mosel and Herrmann (1974), also reported an end of season increase in concentration.

The biosynthetic relationship among these classes of flavonoids has been discussed by Heller and Forkmann (1988) and Stafford (1990) and the evidence for the following biosynthetic scheme in apples has been discussed by Lancaster (1992):

Chalcone
↓
Flavanone
↓
Dihydrokaempferol
↓
Dihydroquercetin → Quercetin → Quercetin glycosides
↓
Leucocyanidin → Epicatechin → Procyanidins
↓
↓
Cyanidin glycosides

This does not account for the synthesis of gallocatechin which, in apples, is the only flavonoid identified with 3′,4′,5′-hydroxylation in the B ring. All other compounds have only 3′,4′-hydroxylation in the B ring and the relationship of gallocatechin to these is unknown. Possible intermediates in this pathway, eg dihydroquercetin were not detected.

The parallel changes in concentration of quercetin glycosides, procyanidins and cyanidin glycosides during fruit growth and maturation of both cultivars suggest a coordinated regulation of flavonoid enzymes in response to a common environmental or developmental signal(s), whilst the stability of the relative proportions of quercetin glycosides also suggests coordinated regulation of glycosyl transferases in apple fruit. Work is currently underway to determine the developmental regulation of activity of enzymes in the pathway to cyanidin glycosides.

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